SYMBIOTIC SOIL FUNGI AS DRIVERS OF SUSTAINABLE NUTRIENT CYCLING

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Sebastian Franz Bender

aus

Deutschland

Promotionskomitee

Prof. Dr. Marcel van der Heijden (Vorsitz)

Prof. Dr. Bernhard Schmid

Dr. Franz Conen

Dr. Jaap Bloem

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„Any fool can make something complicated. It takes a genius to make it simple.“

- Woody Guthrie

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General Introduction

Nutrient cycling in Agriculture

Nitrogen and phosphorus are the two elements essential to life. Although the atmosphere consists of approx. 78 % nitrogen, it is not usable by most organisms in this form. Only a few bacterial and archaeal species possess the capability to bind atmospheric N and transform it into forms available to plants and other organisms (Smil, 1999). Phosphorus is strongly bound in many soils and only a very small part of a soils` P content is available to plants (Bieleski, 1973). Consequently, most naturally occurring plant species, species communities and ecosystems are adapted to low availability and efficient use of these nutrients (Vitousek et al., 1997).

Modern agriculture, as it developed since the early 20th century, however focused on enhanced external inputs of limiting plant nutrients. Since the development of the Haber Bosch process, allowing atmospheric N to be industrially converted into plant available forms, a strong increase in agricultural production had been possible (Gruber and Galloway, 2008). Phosphorus was increasingly won from rock phosphate mines. The high external fertilizer inputs built the basis for the welfare of huge parts of the world and allowed the world population to increase to numbers never achieved before (Galloway et al., 2004). Modern agriculture, and hence the nutrition of huge parts of the global population is strongly dependent on external fertilizer input. However, it is unsure whether the amounts of fertilizer needed in the future, will be available and affordable. Moreover, the strong interference by humans with nutrient cycling also lead to severe environmental impacts. These issues are discussed in the following for nitrogen and phosphorous.

Nitrogen cycling and related problems

Human activities more than doubled the amount of bioavailable N being provided by natural N fixation which had sustained life on earth since thousands of years (Vitousek et al., 1997), but it`s removal from the biosphere did not increase to the same extend (Galloway et al., 2004; Schlesinger, 2009). This has led to an accumulation of reactive N in the biosphere, leading to serious

environmental hazards (Galloway et al., 2003). Human interference with the N cycle is considered one of the severest threats for the resilience of major components of earth-system functioning (Rockstrom et al., 2009).

Of the nitrogen fertilizer applied to agricultural fields, on average 50% are taken up by the crops (Liu et al., 2010; Smil, 1999). The remaining N is potentially prone to getting lost from the soil where it was applied to. Excess N not taken up by crops can enter the "N cascade" (Galloway et al., 2003) and cause severe environmental damage.

Through water movement through the soil profile, e.g. after rainfall, N compounds can enter ground and surface waters where they can lead to water eutrophication (Carpenter et al., 1998). For example the arise of hypoxic and anoxic zones in many coastal regions worldwide unable to sustain most forms of life is directly being attributed to huge N loads originating from agricultural activities being transported through rivers to the sea (Howarth, 2008). Moreover, high concentrations of NO_3^--N in drinking water can damage human health (Ward et al., 2005).

Increased availability of fertilizer N in soil can also promote gaseous losses of reactive N compounds. The microbial mediated processes of nitrification and denitrification can both release the strong greenhouse gas N_2O. N_2O has a relatively long residence time in the atmosphere and has been reported to have a 300 times higher global warming potential as CO_2 (Forster et al., 2007) . It is, after water vapor, CO_2 and Methane the fourth largest contributor to global warming (Socolow, 1999). In addition to this, N_2O is considered the "dominant ozone-depleting substance in the 21st century" (Ravishankara et al., 2009).

N exports from agricultural fields are also a cause of biodiversity loss. In unmanaged ecosystems adapted to relatively low N availability, N input through water and air pathways leads to the dominance of species that can utilize additional N in the most efficient way, hence outcompeting less adapted species (Clark et al., 2007; Socolow, 1999). This species loss might also have consequences for ecosystem functioning (Southon et al., 2013).

The only process that can remove fixed N from the biosphere and, hence, close the N cycle initiated by N_2 fixation is denitrification (Seitzinger et al., 2006). However, the rates of reactive N input to the biosphere are greater as the rates of reactive N removal through denitrification to N_2 (Galloway et al., 2003).

The industrial production of N fertilizer requires high amounts of energy, mostly gained through the combustion of non-renewable fossil fuel resources (Vance, 2001). These resources are however very likely to become limiting within the next few decades (Aleklett et al., 2010). Hence, the availability of sufficient mineral N fertilizer at prices not compromising farm profitability and to ensure agricultural production able to feed a growing human population is questionable (Vance, 2001).

Phosphorous cycling and related problems

Phosphorous is after N the element most frequently limiting plant growth. Phosphate fertilizers have greatly helped to increase agricultural yields and to feed a growing global population in the past century (Gilbert, 2009). In contrast to N, the amount of bioavailable P cannot be enhanced by industrial transformations or symbiotic fixation. Many soils have a moderate to high P-sorption capacity. If phosphate fertilizers are applied to such soils, the phosphate quickly reacts with the soil environment, being adsorbed to mineral clay particles, Fe- or Al- oxides, being precipitated as Fe- Al- or Ca-phosphates or forming organic complexes (Plante, 2007). These P compounds are hardly plant available and only a small part (<1%) of total soil P is usually found in plant available form of orthophosphate in the soil solution. This leads, in some agricultural systems, to amounts of P applied several times higher than the amounts of P exported in products (Simpson et al., 2011; Weaver and Wong, 2011). Consequently, P fertilizer use is highly inefficient in such systems and P is accumulating in soils (Barberis et al., 1995; Frossard et al., 2000). The accumulation of P in soil also enhances the risk of P losses.

When phosphate fertilizer is applied to soils with low P sorption capacity, e.g. deep sandy soils or high organic matter soils, a much higher portion of the P applied is plant available, but also the risk of

P leaching is enhanced (Daly et al., 2001; Ozanne et al., 1961; Weaver et al., 1988). Although, P leaching losses are generally low compared to N leaching, they can be very high in soils with low P sorption capacity (Lewis et al., 1987; Ozanne et al., 1961).

P is commonly the limiting nutrient in freshwater systems. Even economically less relevant P leaching losses may have a strong environmental impact, as P input to freshwater bodies is considered a main cause of water eutrophication, causing severe environmental problems in many parts of the world (Carpenter et al., 1998; Sharpley et al., 2001; Ulen et al., 2007).

Phosphate fertilizers are derived from phosphate rock which is being mined. Phosphate rock is, however, a non-renewable resource. It has been estimated that global phosphate stocks might get depleted within the next 50-100 years, with the quality of the mined material decreasing and mining costs rising (Cordell et al., 2009). The major global phosphate rock reserves are controlled by very few countries, mainly China, Morocco and the USA, making phosphate availability also dependent from political influence. Western Europe is, for example completely dependent on phosphate imports (Cordell et al., 2009).

Sustainability in agriculture

Following the definition of Sustainable development defined by the World Commission on Environment and Development *(Brundtland, 1987),* I define sustainable agriculture as follows:

Sustainable agriculture is agriculture that meets the needs of the present without compromising the ability to meet the needs of future generations. Several aspects of current agricultural practices as conducted in many parts of the world do not match this definition.

First, intensive management of agricultural fields including e.g. monocultures, soil tillage and application of pesticides and high amounts of mineral fertilizers, has strong impacts on the above and belowground biodiversity (Beketov et al., 2013; Helgason et al., 1998; Oehl et al., 2003b; Postma-Blaauw et al., 2010; Robinson and Sutherland, 2002; Verbruggen et al., 2010). There are indications, that soil biodiversity loss has implications for ecosystem functioning (Brussaard et al., 1997; Hooper

et al., 2012; Philippot et al., 2013; Southon et al., 2013; Wagg et al., 2014). Once, species go extinct, the services they may have provided might die out, too, making them unavailable for future generations.

Second, the above mentioned detrimental effects of high inputs of limited fertilizer resources combined with low nutrient use efficiency resulting in nutrient losses causing severe environmental damage represents a major threat for future generations and the earth system as a whole (Rockstrom et al., 2009). Agriculture can meet the needs of the present for huge parts of the global population, hence accomplishing the first part of the definition. But the compliance of the second part, namely preserving the ability of future generations to meet their own needs is highly questionable. The accomplishment of this part of the definition is further endangered by the predicted strong increase of the human population within the next decades (Cleland, 2013).

Agricultural food production as practiced today finds itself in a dead end road. Given that our planet is a closed system with a given amount of resources, there is only one way to achieve sustainability in the long term. It is to use the capital we have in the most efficient way, to provide conditions under which the internal regulations that maintained natural ecosystems for thousands of years can serve the needs for agricultural production.

Biological regulation of N and P cycling

Most nutrient transformations in soil are driven by soil organisms. Through their activities they drive nutrient cycling and determine whether nutrients are made available to plants, are immobilized or are prone to being lost from ecosystems (Philippot et al., 2009; Plante, 2007; Robertson and Groffman, 2007; van der Heijden et al., 2008). The crucial role of soil biota for ecosystem functioning, also in agricultural contexts is increasingly being recognized (de Vries et al., 2013; Mader et al., 2002).

Arbuscular mycorrhizal fungi

One group of soil organisms with particular importance to many ecosystems are arbuscular mycorrhizal (AM) fungi. Arbuscular mycorrhizal fungi are a group of soil microorganisms that are dispersed worldwide and live in symbiosis with 70-90 % of all land plant species including many agricultural crops like corn, wheat, soya, rice or potatoes (Smith and Read, 2008). AM fungi colonize plant roots, where they form characteristic structures inside the plant cells called arbuscles, believed to be the main site of nutrient and carbon exchange between the fungus and plant. AM fungi connect the host plants to their hyphal network in soil, which can reach densities higher as 100m of hyphae per cm^3 soil (Miller et al., 1995). Hence, AM fungi make up a significant portion of the total soil microbial biomass (Olsson et al., 1999).

It has been estimated that 20% of photoassimilated C by the plants is transferred to AM fungi (Jakobsen and Rosendahl, 1990), providing an essential C source for the survival of the fungus (Parniske, 2008). AMF can acquire nutrients from soil, transfer them to their host plants and improve plant nutrition (George et al., 1995; Smith and Read, 2008). Exploration of a larger soil volume through the extraradical hyphal network and efficient nutrient uptake is considered one of the key mechanisms for the improvement of plant P nutrition through AMF (Jakobsen et al., 1992; Jansa et al., 2005) Sanders and Tinker, 1971.

AM fungi were also shown to transfer N from soil to plant and in some (Atul-Nayyar et al., 2009; Cavagnaro et al., 2012; George et al., 1995), but not all (Ames et al., 1983; Hawkins et al., 2000; Reynolds et al., 2005) cases improve plant N nutrition. The relevance of AM fungi on plant N nutrition under ecological relevant conditions is still unclear (Fitter et al., 2011).

While a substantial body of research on AM fungal effects on plant nutrition and performance exists, their involvement in other ecosystem processes has received relatively little attention (Rillig, 2004).

Little is known about the influence of the AMF symbiosis on the overall biogeochemistry of ecosystems, especially how the cycling of N and P is affected. It has been shown that enhanced uptake of mineral nutrients by AM fungi can deplete soil inorganic nutrient pools (Johansen et al.,

1993; Li et al., 1991). Moreover, there is evidence that AM fungi enhance the mineralization of organic matter and the subsequent uptake of released mineral nutrients (Atul-Nayyar et al., 2009; Hodge et al., 2001; Jayachandran et al., 1992). AM fungi can also directly take up organic N compounds (Whiteside et al., 2012; Whiteside et al., 2009). It has also been shown, that AMF effectively scavenge for P and can deplete soil mineral P pools (Li et al., 1991) and utilize organic (Jayachandran et al., 1992) and insoluble P compounds like iron phosphates (Bolan et al., 1987).

These results indicate, that AM fungi might have profound influences on P and N cycling exceeding effects on plant nutrition. Effective nutrient uptake should reduce the risk of nutrient losses from soil. Furthermore, by affecting soil nutrient availability, AM fungi are also likely to affect nutrient availability for other soil biota and, hence, the nutrient transformation processes they perform.

A limited number of studies has, so far, experimentally addressed the ability of AM fungi to reduce nutrient leaching losses (Asghari and Cavagnaro, 2011; Asghari and Cavagnaro, 2012; Asghari et al., 2005; Corkidi et al., 2011; van der Heijden, 2010). These greenhouse studies show, that AM fungi possess the capability to reduce the leaching of important plant nutrients. However, the results obtained there seem to be variable and context dependent. The ecological relevance of AMF to reduce nutrient leaching is still unclear. The knowledge about the effects of AMF on nutrient leaching has to be increased by assessing whether AMF can reduce overall nutrient loss, or just the loss of certain nutrient compounds, and by assessing the ability to reduce nutrient leaching under various environmental conditions and under ecological relevant conditions.

While there are indications that AM fungi affect soil microorganisms involved the production of N gases, e.g. through denitrification (Ames et al., 1984; Veresoglou et al., 2012b), it remains unknown whether this also affects the processes performed by these organisms, e.g. N_2O and N_2 production.

To completely understand the effects of AMF on nutrient cycling in plant soil systems, a comprehensive assessment of the nutrient distribution among soil, plant, groundwater and atmosphere is required.

There is evidence to suggest that AMF may have the potential to greatly enhance the sustainability of agricultural systems by improving plant nutrition, effectively taking up nutrients from soil, reducing nutrient losses and, hence, increasing the nutrient use efficiency and reducing the amount of fertilizer required to obtain satisfactory plant yields. Figure 1 presents a conceptual Framework of the potential benefits a functioning soil community including AM-fungi might have on the sustainability of agricultural systems.

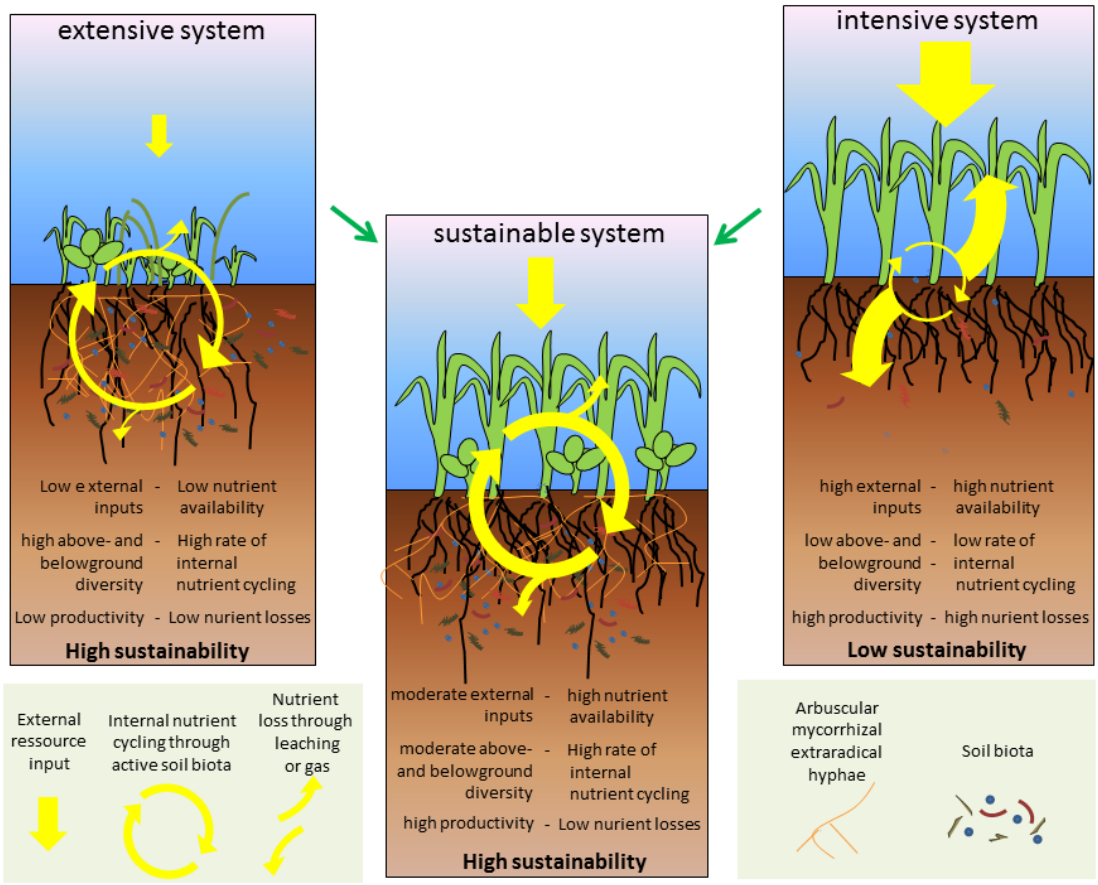

Figure 1: Conceptual model showing the relation of resource inputs, -losses and internal cycling performed by soil biota in relation to management intensity. The extensive system has a rich soil life and is characterized by low resource inputs and outputs, a high rate of internal nutrient cycling and low productivity. The intensive system has a depleted soil life, is characterized by high resource inputs, high losses, a low rate of internal nutrient, but high productivity. The sustainable system presents a trade-off between the other two systems, has a rich soil life and is characterized by moderate resource inputs, a high rate of internal nutrient cycling, low nutrient losses and high productivity.

Thesis Outline

The research performed during this PhD thesis had the objective to elucidate the role the AM fungal symbiosis plays in nutrient cycling and, especially, if they are able to reduce nutrient losses from soil. The overall aim was, to determine whether AM fungi can contribute to sustainable nutrient cycling. This knowledge could then be used to improve the nutrient efficiency and sustainability of cropping systems by adopting management practices favorable for AM fungi. The most important questions were (1) Can AMF reduce nutrient leaching from soil?, (2) Do AMF affect gaseous emissions of N via denitrification?, and (3) Can AMF contribute to sustainable agricultural practices by improving plant nutrition and reducing nutrient losses?

During my PhD work, I conducted 4 experiments, all investigating the role of AM fungi in nutrient cycling and especially in reducing nutrient losses.

In Chapter 1, I address the question whether AM fungi can affect greenhouse gas emissions from soil. In two independent greenhouse experiments using two different approaches to manipulate the presence of AM fungi, we measured emissions of the important greenhouse gas N_2O after a fertilization pulse in model-ecosystems with and without AM fungi. We provide the first evidence, that the AM fungal symbiosis is capable of reducing denitrification-related emissions of N_2O.

In Chapter 2, I provide an comprehensive assessment of the AM fungal influence on N and P cycling in experimental grassland with varying environmental conditions, e.g. different soil types and fertilizer compositions. AM fungi enhanced plant P contents, reduced P leaching losses and increased P mobilization from soil P resources. While AM fungi consistently reduced emissions of N_2O, the effects on plant N nutrition and N leaching varied between soil types. The results show that the effects of AM fungi on P cycling seem to be consistent among different environmental conditions, while effects on N cycling seem to be more context dependent.

In chapter 3, I investigated the complete N cycle of a plant-soil system. We assessed the N distribution among plant and soil, N leaching losses and complete denitrification losses, including measurements of N_2, in dependence of AM fungi. The results indicate, that AMF greatly can enhance

the sustainability of plant soil systems by enhancing plant N nutrition, reducing N leaching, reducing N_2O emissions and, apparently increasing the emissions of N_2. As N_2 production from denitrification is the only process globally that can transform reactive N back into unreactive, atmospheric N_2, these results suggest, that AMF might open new perspectives for global N cycling and sustainability.

While the experiments in the first three chapters were all performed in the greenhouse, in chapter 4, an outdoor lysimeter experiment was conducted in an agricultural context. We filled lysimeters of 230 L volume with sterilized soil in two horizons and inoculated them either with a microbial control inoculum, or a soil biota inoculum containing AMF and other groups of soil organisms <2 mm. An agricultural crop-rotation was planted and plant yield and nutrition, as well as nutrient leaching losses were analyzed for a period of almost two years. The results obtained clearly show that soil biota play a substantial role for the sustainability of agricultural cropping systems and highlight the necessity to apply management practices that favor soil life in order to achieve sustainable cropping systems with a high nutrient use efficiency and low environmental impact.

In the General Discussion, the results obtained in the chapters 1-4 are summarized and discussed within the context of sustainable agriculture.

Chapter 1

Symbiotic relationships between soil fungi and plants reduce N_2O emissions from soil

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Abstract

N_2O is a potent greenhouse gas involved in the destruction of the protective ozone layer in the stratosphere and contributing to global warming. The ecological processes regulating its emissions from soil are still poorly understood. Here we show, that the presence of arbuscular mycorrhizal fungi (AMF), a dominant group of soil fungi, which form symbiotic associations with the majority of land plants and which influence a range of important ecosystem functions, can induce a reduction in N_20 emissions from soil. To test for a functional relationship between AM fungi and N_2O emissions, we manipulated the abundance of AMF in two independent greenhouse experiments using two different approaches (sterilized and re-inoculated soil and non-mycorrhizal tomato-mutants) and two different soils.

N_2O emissions were increased by 42 and 33% in microcosms with reduced AMF abundance compared to microcosms with a well-established AMF community, suggesting that AMF regulate N_2O emissions. This could partly be explained by increased N immobilization into microbial or plant biomass, reduced concentrations of mineral soil N as a substrate for N_2O emission, and altered water relations. Moreover, the abundance of key genes responsible for N_2O production (*nirK*) was negatively and for N_2O consumption (*nosZ*) positively correlated to AMF abundance, indicating that the regulation of N_2O emissions is transmitted by AMF-induced changes in the soil microbial community. Our results suggest that the disruption of the AMF symbiosis through intensification of agricultural practices may further contribute to increased N_2O emissions.

Introduction

N_2O is a potent greenhouse gas contributing to global warming with a 300 times higher global warming potential than CO_2 and is involved in the destruction of the protective ozone layer in the stratosphere (Forster et al., 2007; Ravishankara et al., 2009). N_2O has, after CO_2 and CH_4, the highest impact on the greenhouse effect and its importance is expected to increase due to its longevity and a predicted increase in future emissions (Montzka et al., 2011). Approximately 57% of global N_2O emissions are thought to derive from terrestrial soils (Mosier et al., 1998). A major process producing N_2O in soils is denitrification, a microbial respiratory process that reduces nitrogen oxides (NO_3^-, NO_2) to the gaseous products N_2O and N_2 when oxygen is limiting (Philippot et al., 2007; Seitzinger et al., 2006). It is well established that denitrification depends on soil nitrogen and carbon substrate availability and quality, soil water content, pH and temperature (Knowles, 1982). However, the knowledge of ecological interactions among the vast variety of soil biota on denitrification and N_2O emissions is mostly limited to effects of earthworms and nematodes (Djigal et al., 2010; Lubbers et al., 2013), while effects of other soil invertebrates on N_2O emissions are just recently being discovered (Kuiper et al., 2013).

A potential effect of arbuscular mycorrhizal fungi (AMF) on N_2O emissions has been hypothesized (Cavagnaro et al., 2012; Veresoglou et al., 2012a), but has, to our knowledge, never been thoroughly tested. This is surprising because AM fungi associate with two thirds of all land plants and are among the most abundant functional groups of soil microorganisms being present in almost any ecosystem investigated. They are obligate plant symbionts and are known to improve plant nutrition and influence plant diversity and ecosystem functioning (Cheng et al., 2012; Smith and Read, 2008; van der Heijden, 2010; van der Heijden et al., 1998).

AMF induce changes in soil structure and soil aggregation (Rillig and Mummey, 2006), soil water relations (Auge, 2001), pH (Bago et al., 1996), and in the availability and quality of labile carbon (Graham et al., 1981; Hooker et al., 2007), all being factors affecting denitrification. Several studies also show that AMF influence bacterial communities inhabiting the rhizosphere and

mycorrhizosphere (Ames et al., 1984; Scheublin et al., 2010), including shifts in denitrifying communities (Amora-Lazcano et al., 1998; Veresoglou et al., 2012b). AMF influence the N cycle and can take up significant amounts of nitrogen (Hodge and Fitter, 2010; Veresoglou et al., 2012a). By reducing the availability of soluble N in the soil, AMF could also reduce denitrification and N_2O emission rates. Thus, there is compelling evidence to suggest, that AMF influence denitrification.

It has been shown that fungi possess the ability to denitrify and that fungal N_2O emissions through denitrification can be of high ecological relevance (Herold et al., 2012; Laughlin and Stevens, 2002; Shoun et al., 1992), but we know of no study reporting denitrifying ability for arbuscular mycorrhizal fungi.

To test for a functional relationship between AMF abundance and N_2O emissions, we conducted two independent greenhouse experiments with differing approaches and soils. It was hypothesized, that (i) a reduced abundance of AMF increases denitrification related emissions of N_2O, (ii) an increase in emissions is driven by a reduction in plant and/or microbial biomass N pools and (iii) is related to alteration in abundance of key genes for denitrification.

Material and methods

Two experiments (the “grass-experiment” and the “tomato-experiment”, see below for details) were conducted in microcosms constructed from PVC tubes with a diameter of 15cm, a height of 40cm, and a volume of approx. 7l. Each microcosm had a removable, airtight cap, allowing the headspace to be closed for gas measurements (see Fig.S1 for details).

Grass-experiment

The soil was collected from a long-term grassland site at the Research Station Agroscope ART in Zürich, Switzerland (47°42`78.13`` N, 8°51`78.38`` E). It was a slightly acid brown earth with a sandy-loam texture. The collected soil was 5mm sieved, air dried, and mixed with quartz sand to a soil:sand-ratio of 7:3 (v/v). The mixture was gamma irradiated with a maximum dose of 32 kGy to eliminate indigenous AMF. After irradiation, soil was incubated at room temperature for 4 weeks to allow

stabilization of soil chemical properties before the experiment was initiated. The experiment consisted of two treatments, the mycorrhizal (M) treatment and the non-mycorrhizal (NM) treatment, each being replicated 10 times and set up in three randomized blocks. Each microcosm was filled with 5000ml of the sterilized soil and 270ml of a inoculum-mixture of three common AMF species; the NM- microcosms received a non-mycorrhizal control inoculum. Inoculum details are given in Supplementary information. Soil irradiation not only eliminated indigenous AMF but will also have removed a significant proportion of other soil biota. Therefore, to include microbes from natural grassland and to allow a similar microbial background among the AMF and control inoculums, a microbial wash was mixed into the substrate for each microcosm (Koide and Li, 1989; van der Heijden et al., 2006). The microbial wash was produced from the same fresh field soil used to fill the microcosms and from all inocula used in the experiment. In addition, 400ml sterilized soil-sand mixture was added on top of the microcosms to reduce the risk of contamination between pots. Seeds of *Lolium multiflorum* var. oryx were surface-sterilized by stirring in 1,25% bleach for ten minutes and rinsing them with deionized water. They were allowed to germinate on 1.5% water agar for one week, before planting 30 evenly spaced seedlings into each microcosm. After planting, pots were transferred to a climate chamber with a 16h, 22°C day, light intensity of 200µmol m^{-2} sec^{-1}, and an 8h, 16°C night. Relative humidity was 65% at day and 85% at night. Microcosms were watered regularly by weight with deionized water to 40% water filled pore space (WFPS). Plant shoots were cut 6 weeks after planting, approximately 3cm above soil surface, and were allowed to re-grow. The experiment was started on November 5, 2010.

Tomato-experiment

The soil was collected from a regularly manured long-term pasture on a calcareous brown earth with a sandy-loam texture of an organic farm near the Research Station Agroscope ART in Zürich, Switzerland (47°43`11.83`` N, 8°53`65.25`` E). The soil was sieved through a 5 mm sieve to homogenize and to remove large stones, plant material, earthworms and other macrofauna that could cause undesired variation. Microcosms were filled with 6000 ml of the sieved field soil. In

addition to this, 550 ml of an additional AMF inoculum was mixed with this soil to assure a high AMF root infection potential. Inoculum details are given in Supplementary information. Hyphal bags made from 30µm nylon mesh and filled with 40g autoclaved quartz sand were buried approximately 5 cm below soil surface. The fine mesh prevented roots from entering the bag, but allowed AMF hyphae to pass. Two genotypes of Tomato (*Solanum lycopersicum L.* cv. Micro-Tom), the BC1-mutant and its progenitor wild-type, were planted into the microcosms. The BC1-mutant exhibits a strongly reduced AMF root colonization compared to its` wildtype progenitor (Meissner et al., 1997). This mutant/wildtype pair was created by fast-neutron mutagenization (David-Schwartz et al., 2001) and hybridization and has been demonstrated to be very suitable for studies in AMF ecology (Rillig et al., 2008). The tomato seeds were germinated in a sterilized 1:1 (v/v) sand-soil mixture and then transplanted into the microcosms. A test for equal performance of both tomato genotypes in absence of AMF was conducted and is described in the Supplementary information (Table S1).

The plants were grown in a greenhouse with an average daily temperature of 24 ºC, nightly temperature of 18 ºC and 16 hours of light per day. Supplemental light was provided by 400 W high-pressure sodium lights when natural irradiation was lower than 300W/m^2. Plants were regularly watered to 40% WFPS with deionized water. The tomato-experiment consisted of two treatments, the M treatment planted with the wildtype, and the NM treatment planted with the BC1-mutant, each replicated 10 times and was established in three randomized blocks. One replicate of the NM treatment failed and was irretrievably lost. The blocks were set up in two week intervals, starting July 26, 2011.

In the field, both soils used in this study are regularly subjected to waterlogging under wet weather conditions. The characteristics of the substrates being filled into the microcosms of both experiments are summarized in Table S2. When filling the microcosms, substrate dry weights were determined gravimetrically. The exact weight of the pots was noted to be able to calculate the WFPS as described in the Supplementary information.

Fertilization and Water Pulse

In the grass-experiment, after 13 and 14 weeks of plant growth, each pot received 10 ml of a nutrient solution with a low NO_3^--N concentration (9.98mM KNO_3, 1mM $MgSO_4$, 1.5mM KH_2PO_4, 2mM $CaCl_2$, 50µM KCl, 25µM H_3BO_3, 2µM $MnSO_4$, 2µM $ZnSO_4$, 0.5µM $CuSO_4$, 0.5µM Na_2MoO_4). After 15 weeks, microcosms were watered to 100% WFPS with deionized water mixed with 10 ml of a nutrient solution (778mM KNO_3, 59mM KH_2PO_4, 1mM $MgSO_4$, 2mM $CaCl_2$, 50µM KCl, 25µM H_3BO_3, 2µM $MnSO_4$, 2µM $ZnSO_4$, 0.5µM $CuSO_4$, 0.5µM Na_2MoO_4). This corresponded to a fertilizer pulse of 60kgN/ha and 10kgP/ha. The higher water and nutrient loadings were introduced to provide conditions conducive for denitrification.

In the tomato-experiment, after 10 weeks of plant growth, the microcosms were watered to 94% WFPS with deionized water mixed with 10ml of nutrient solution as applied in the grass-experiment after 15 weeks. After fertilization, gas fluxes were measured.

Gas sampling

To measure the fluxes of N_2O and CO_2 from the microcosms, the headspace was adjusted to a height of 20cm above soil surface (4l volume) and closed for a period of 10 minutes with the headspace gas pumped through a sample loop, first into a LI-820 CO_2 Gas Analyzer (LI-COR Biosciences, Lincoln, US) and, subsequently, to a TEI46c automated N_2O analyzer (Thermo Fisher Scientific, Waltham, US). The cap used to close the headspace was non-transparent. At every gas sampling, the respective pot was weighed to determine the actual WFPS.

In the grass-experiment, after fertilization, lights remained on to avoid diurnal variation in gas fluxes. Headspace gas was analyzed for CO_2 and N_2O emissions at approximately every 6h for 72 hours and once at 89h after the fertilization pulse, resulting in 13 flux measurements per microcosm.

In the tomato-experiment, gas fluxes were measured three times per day (morning, noon and evening) starting 24h after fertilization for 6 days, and once at the 7th day (noon), resulting in 19 flux measurements per microcosm.

Harvest

Before the final harvest and after the gas measurements, microcosms in both greenhouse experiments were watered, received an artificial rainfall and leachates were collected as described in van der Heijden (2010). Shoots were cut at the soil surface. The microcosms were emptied and the roots where collected thoroughly from soil, rinsed with water, cut into pieces <2cm, and a subsample was weighed and stored in 50% Ethanol. Shoots and remaining roots were dried at 60°C, and weighed. In the tomato-experiment, the hyphal bags were extracted and frozen for real time PCR analyses. The remaining substrate was mixed thoroughly and soil samples taken for soil analyses and assessment of AMF extraradical hyphal length.

Analyses

Soil, leachate and plant samples were chemically analyzed and AMF root colonization and extraradical hyphal length determined as described in Supplementary information.

Gene copy numbers

To test, if AMF affect the bacterial communities involved in denitrification, we quantified copy numbers of key genes involved in denitrification and N_2O production, encoding cd1 and copper nitrite reductases (*nir*S and *nir*K) and nitrous oxide reductase (*nos*Z) (Zumft, 1997) from hyphal bag samples in the tomato-experiment. Bacterial *16S rRNA* gene abundance was determined to assess the size of the total bacterial community in the samples.

Gene copy number estimations were performed using relative real time estimation against a reference target to increase accuracy and sensitivity of detection (Daniell et al., 2012). Briefly, DNA was extracted from hyphal bag samples by a modified phenol chloroform extraction method with bead beating (Deng et al., 2010) with the addition of the reference target. Bacterial *16S*, reference target and denitrification gene amplification was performed essentially as described in Daniell et al. (2012) with the primer pairs and reaction conditions shown in Table S3. All amplifications were performed using the SYBR green I master mix (Roche, Burgess Hill UK) with the recommended

conditions and 10 pmol μl^{-1} of each primer on a lightcycler 480 (Roche) with associated relative quantification software with three technical replicates performed per sample.

Statistical analyses

Repeated gas-flux measurements were analyzed using the mixed procedure in SPSS version 20 (IBM corp., Armonk, NY, US). This approach uses the Satterthwaite approximation to obtain the degrees of freedom (Satterthwaite, 1946). The linear mixed effect models for N_2O and CO_2 fluxes included measurement time, AMF treatment and the interaction as fixed effects, and the measurement time nested within each microcosm as the repeated compound. The repeated measurements taken on the same pot were assumed to be correlated. We fit several models using different correlation structures. The adequate correlation structure was chosen by minimizing Akaike information criterion (AIC) and performing Log-likelihood tests. To reduce calculation effort in the tomato-experiment, the repeated measurements taken on the same day were averaged. This reduced the number of repeated measures from 19 to 7. Cumulative gas emissions were calculated by linear interpolation between measurements. Plant biomass and N content, soil data, WFPS, Microbial biomass C and N contents, their molar ratio and AMF parameters were statistically analyzed using linear mixed effects models with the AMF treatment as factor and the Block as random effect. Non-parametric Kruskal-Wallis tests were performed to test the differences in AMF parameters between treatments in the grass-experiment. Gene copy numbers of denitrification genes and their ratio in the tomato-experiment were analyzed similarly but the three technical replicates were nested within each individual pot. Pearson-correlations of AMF parameters with N_2O emissions, microbial biomass and gene copy numbers and their ratio were performed. Data was checked for normality and homogeneity and log-transformed where necessary.

For the tomato-experiment, a multiple regression was performed to identify the most influential pathways, by which the presence of AMF affected N_2O emissions as described in Table S4. As no gene copy number data was available, no multiple regression was performed for the grass-experiment. All

statistical analyses except for gas-fluxes were done using the software R version 2.14.1 and the R-package “nlme” (Pinheiro et al., 2011).

Results

Grass-experiment

Gas emissions

Immediately after fertilization and watering, the N_2O emission curves in both treatments increased in the grassland microcosms (Fig.1a). After this initial phase, N_2O fluxes varied significantly between the treatments (time:AMF interaction $F_{12,18.03}$=8.65, P<0.001, see Table 1a). The peak of N_2O flux was both attained earlier and was lower in the M treatment compared to the NM treatment (Fig.1a). Cumulatively, N_2O emissions were 42.4% higher in microcosms without AMF compared to mycorrhizal microcosms. Emissions of CO_2 also differed significantly between treatments (time:AMF interaction $F_{12,15.35}$=3.88, P<0.007, Table 1a, Fig.2a). Cumulative CO_2 emissions were reduced by 5% in the NM treatments.

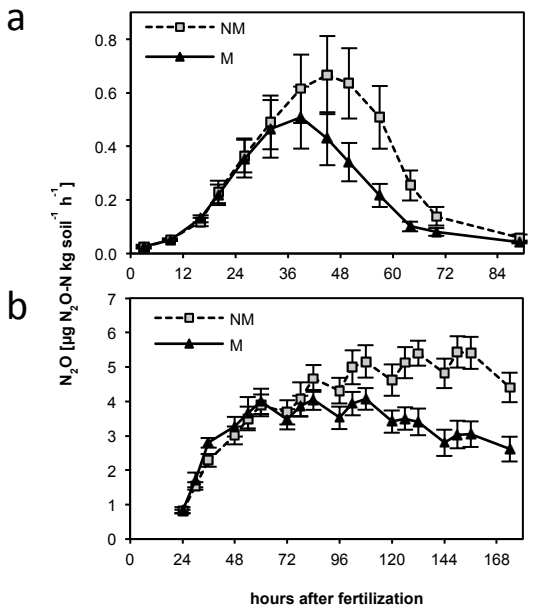

Figure 1: N_2O fluxes from mycorrhizal (M) and non-mycorrhizal (NM) microcosms after a water-and fertilization pulse corresponding to 60 kg N/ha in the grass-experiment (a), and the tomato-experiment (b). Grey squares and dashed line: non-mycorrhizal treatment (NM); black triangles and solid line: mycorrhizal treatment (M). Error bars = ±1SEM (n=10 for the grass-experiment; for the tomato-experiment, n=9 for the NM, and n=10 for the M treatment).

Plant and soil measures

There were no significant differences between the treatments in plant biomass and N nutrition and soil N content and pH at the end of the experiment (Table 2). The water content, expressed as the reduction in WFPS during the gas measurements, did also not reveal any differences (Table 2, Fig.S2). Roots from the NM treatments did not show any colonization with AMF structures. However some extraradical hyphae were detected in the NM treatment. Those were considered as non-mycorrhizal or dead fungal hyphae.

Soil microbial biomass C and N contents were significantly increased in the M treatment (Table 2). There was a positive correlation (R^2=0.67, P=0.004) of AMF extraradical hyphal length with soil microbial biomass N (Fig. 3).

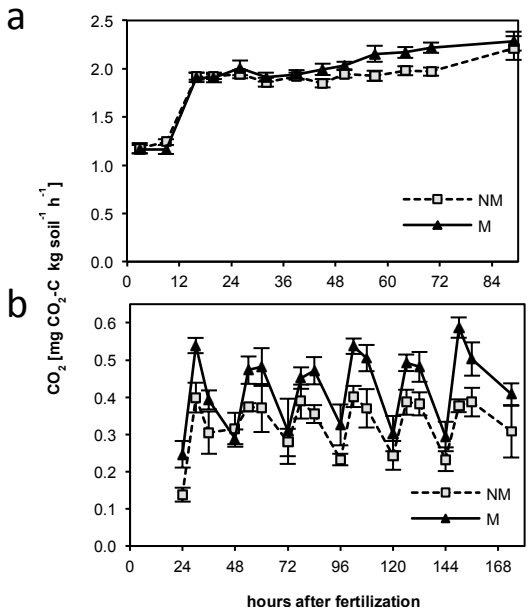

Figure 2: CO_2 fluxes from mycorrhizal (M) or non-mycorrhizal (NM) microcosms after a water-and fertilization pulse in the grass-experiment (a), and the tomato-experiment (b). The measurements in the grass-experiment were made in a climate chamber with lights constantly switched on during the whole measuring period. In contrast, in the tomato-experiment, measurements were made in a greenhouse with a 16h day/ 8 night pattern. This resulted in pronounced diurnal CO_2 flux variations in the tomato-experiment, while no such pattern was detected in the grass-experiment. Grey squares and dashed line: non-mycorrhizal treatment (NM); black triangles and solid line: mycorrhizal treatment (M). Error bars represent ± 1 SEM (n=10 for the grass-experiment; for the tomato-experiment, n=9 for the NM, and n=10 for the M treatment).

Table 1: ANOVA-output of the repeated measures analysis for the N_2O and CO_2 fluxes in the grass-experiment (a) and the tomato-experiment (b).

a

Grass-Experiment

response	**log(N_2O)**			
Variable	num *df*	den *df*	*F*-value	*P*-value
(Intercept)	1	18.00	87.06	<0.0001
time	**12**	**18.03**	**23.06**	**<0.0001**
AMF	1	18.00	1.70	0.209
time:AMF	**12**	**18.03**	**8.65**	**<0.0001**
response	**CO_2**			
Variable	*num df*	*den df*	*F*-value	*P*-value
(Intercept)	1	17.98	6094.03	<0.0001
time	**12**	**15.35**	**262.90**	**<0.0001**
AMF	1	17.98	2.52	0.130
time:AMF	**12**	**15.35**	**3.88**	**0.007**

b

Tomato-Experiment

N_2O				
Variable	num *df*	den *df*	*F*-value	*P*-value
(Intercept)	1	17	406.99	<0.0001
time	**6**	**17**	**59.98**	**<0.0001**
AMF	**1**	**17**	**6.71**	**0.019**
time:AMF	**6**	**17**	**5.35**	**0.003**
CO_2				
Variable	num *df*	*den df*	*F*-value	*P*-value
(Intercept)	1	17.49	512.69	<0.0001
time	**6**	**14.32**	**3.70**	**0.020**
AMF	**1**	**17.49**	**7.07**	**0.016**
time:AMF	6	14.32	0.61	0.716

Abbreviation: AMF, Arbuscular mycorrhizal fungal- treatment

In the grass-experiment, the factor time consisted of 13 levels. In the tomato-experiment, time comprised 7 levels (19 timepoints averaged to 1 mean per day, see Material and Methods for detailed description). The factor AMF consisted of two levels, mycorrhizal (M) and non-mycorrhizal (NM). (n=10 for the grass-experiment; for the tomato-experiment, n=9 for the NM, and n=10 for the M treatment). Significant effects (*P*<0.05) are shown in bold.

Table 2: Plant, soil, and AM fungal parameters of the microcosms being inoculated with (M) or without (NM) AM fungi (grass-experiment) or being planted with a mycorrhizal tomato-wildtype (M) or the non-mycorrhizal BC1 tomato-mutant (NM) (tomato-experiment).

	Grass-experiment					Tomato-experiment				
	M		NM			M (WT)		NM (MT)		
Plant biomass										
Shoot (g kg soil^{-1})	3.025	(±0.035)	3.115	(±0.040)	*P*=0.271	**0.746**	**(±0.043)**	**0.544**	**(±0.039)**	***P*=0.002**
Root (g kg soil^{-1})	3.757	(±0.235)	4.079	(±0.337)	*P*=0.691	0.101	(±0.007)	0.102	(±0.012)	*P*=0.859
Total (g kg soil^{-1})	6.782	(±0.246)	7.194	(±0.334)	*P*=0.613	**0.847**	**(±0.042)**	**0.646**	**(±0.044)**	***P*=0.002**
Plant N content										
Shoot (mgN kg soil^{-1})	68.10	(±1.49)	68.96	(±0.91)	*P*=0.722	**29.69**	**(±1.40)**	**19.98**	**(±1.40)**	***P*<0.001**
Root (mgN kg soil^{-1})	48.05	(±1.64)	52.82	(±4.25)	*P*=0.273	2.613	(±0.306)	2.476	(±0.327)	*P*=0.871
Total (mgN kg soil^{-1})	116.1	(±2.3)	122.8	(±4.5)	*P*=0.213	**32.30**	**(±1.38)**	**22.46**	**(±1.43)**	***P*<0.001**
Soil										
available NO_3^- (gNO_3^--N kg soil^{-1})	nd		nd			**31.05**	**(±1.44)**	**39.05**	**(±1.27)**	***P*<0.001**
Total soil N (gN kg soil^{-1})	0.863	(±0.022)	0.821	(±0.030)	*P*=0.558	2.390	(±0.041)	2.438	(±0.032)	*P*=0.331
Soil pH	7.373	(±0.033)	7.335	(±0.025)	*P*=0.531	**7.745**	**(±0.027)**	**7.636**	**(±0.027)**	***P*=0.004**
WFPS (% reduction h^{-1})	-0.310	(±0.007)	-0.309	(±0.009)	*P*=0.901	**-0.083**	**(±0.003)**	**-0.069**	**(±0.003)**	***P*=0.004**
Soil Microbial Biomass										
C content (mg kg soil^{-1})	**200.8**	**(±5.96)**	**180.2**	**(±6.19)**	***P*=0.028**	743.8	(±10.39)	757.6	(±18.09)	*P*=0.517
N content (mg kg soil^{-1})	**29.98**	**(±1.15)**	**26.26**	**(±1.23)**	***P*=0.026**	107.75	(±1.96)	103.37	(±2.31)	*P*=0.116
C/N ratio	6.75	(±0.227)	6.91	(±0.164)	*P*=0.434	**8.06**	**(±0.129)**	**8.57**	**(±0.204)**	***P*=0.037**
AM fungal parameters										
HC (%)	**64.53**	**(±2.37)**	**0**	**(±0.00)**	***P*<0.001**[a]	**40.9**	**(±3.04)**	**16.1**	**(±2.69)**	***P*<0.001**
VC (%)	**4.80**	**(±0.71)**	**0**	**(±0.00)**	***P*<0.001**[a]	**1.5**	**(±0.307)**	**0.4**	**(±0.221)**	***P*<0.001**
AC (%)	**23.67**	**(±2.16)**	**0**	**(±0.00)**	***P*<0.001**[a]	**34**	**(±2.6)**	**8.3**	**(±1.5)**	***P*<0.001**
HL (m g soil^{-1})	**16.54**	**(±1.19)**	**4.387**	**(±0.28)**	***P*<0.001**[a]	6.78	(±0.519)	5.90	(±0.318)	*P*=0.170
gene copy numbers										
16srRNA (mg soil^{-1})	na		na			34242	(±12146)	23347	(±7460)	*P*=0.412
nirK (mg soil^{-1})	na		na			109.13	(±24.62)	198.13	(±53.98)	*P*=0.105
nirS (mg soil^{-1})	na		na			6.45	(±2.45)	3.36	(±0.72)	*P*=0.282
nosZ (mg soil^{-1})	na		na			64.14	(±10.99)	39.85	(±7.08)	*P*=0.185
ratio *nosZ*/(*nirS*+*nirK*)	na		na			1.37	(±0.739)	0.28	(±0.058)	*P*=0.169

Abbreviations: WFPS, water filled pore space; AMF, arbuscular mycorrhizal fungi; HC, hyphal colonization; VC, vesicular root colonization; AC, arbuscular root colonization; HL, extraradical hyphal length

Values are presented as means (±1 SEM), *P*-values are obtained from linear mixed effects models with the AMF treatment as factor and the Block as random effect. Available NO_3^-- was calculated as the sum of soil and leachate NO_3^- contents at the end of the experiment. In the grass-experiment, no available NO_3^- was detected, in the tomato-experiment on average 62.5 % (±1.93 % SEM) of available NO_3^- was found in the leachate. The WFPS declined nearly linearly during the gas measurements (see Fig. S2) and the slope of the decline in WFPS over time (% reduction in WFPS*h^{-1}, "rWFPS") was used to analyze differences between the treatments. Significant differences between the NM and the M treatments are shown in bold (n=10 for the grass-experiment; for the tomato-experiment, n=9 for the NM, and n=10 for the M treatment).

nd, not detectable. na, not available

[a] a non-parametric kruskal-wallis test was performed to test for differences between treatments

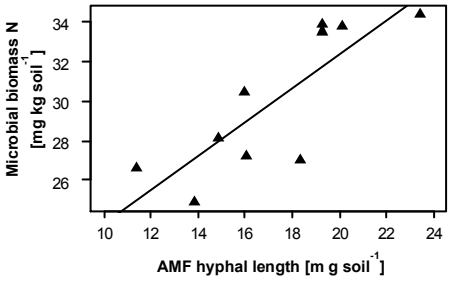

Figure 3: Pearson-correlations of AM extraradical hyphal length with microbial biomass N content (R^2=0.67, *P*=0.004) in the M treatment of the grass-experiment. The NM treatment was omitted from the correlation analysis, as it did not contain AMF.

Tomato-experiment

Gas emissions

N_2O emissions differed significantly between treatments (AMF $F_{1,17}$=6.71, *P*=0.019; time:AMF interaction $F_{6,17}$=4.24, *P*=0.003, Table 1b, Fig.1b). Total N_2O emissions were 33.8% higher in the microcosms planted with the non-mycorrhizal tomato mutant compared to the mycorrhizal wildtype. Similar to the grass-experiment, the peak of N_2O fluxes was reached earlier and was lower in the M treatment (Fig.1b). There was a significant, negative correlation of AMF root colonization to N_2O emissions (R^2= 0.47, *P*=0.001, Fig.4).

CO_2 emissions differed significantly between treatments (AMF $F_{1,17.49}$=6.71, *P*=0.016, Table 1b, Fig.2b). Cumulative CO_2 emissions were 23.4% lower in the NM treatment compared to the M treatment.

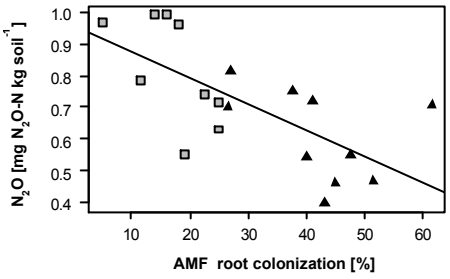

Figure 4: Pearson-correlation of AMF root colonization with N_2O emissions in the tomato-experiment (R^2=0.47, P=0.001). Grey squares: tomato mutant (NM), black triangles: tomato wildtype (M)

Plant and soil measures

NM plants had a 23.7% lower biomass and 30.5% lower N content than M plants. However, root N contents did not differ significantly between the treatments (Table 2).

Available NO_3^- was 25.8% higher in the NM treatment at the end of the experiment, while soil pH was slightly but significantly reduced (Table 2). The water content during the gas measurements declined faster in the M treatment (Table 2, Fig.S2).

Microbial biomass C and N contents did not differ between the treatments. However, the C/N ratio of the soil microbial biomass was significantly higher in the NM treatment (Table 2).

The BC1 mutant did not completely suppress root colonization by AMF, but reduced it significantly. The average root length colonized by AMF were 40.9% and 16.1% for the M and the NM treatment, respectively. Extraradical hyphal length did not differ significantly between the treatments (Table 2).

To exclude the possibility of any non-target effects resulting from differences between the genotypes independent of AMF, a test for equal performance of the genotypes in the absence of AMF was conducted; this demonstrated no significant differences between genotypes in all measured variables (Table S1).

Denitrificaton gene copy numbers

Copy numbers of *nirK*, *nirS* and *nosZ*, key genes involved in denitrification and N_2O production or consumption, and the ratio of nosZ/(nirK+nirS) did not differ significantly between treatments, but AMF parameters were significantly negatively correlated to the copy numbers of the functional gene *nirK* (Fig.5a-b, Table S5). Simultaneously, gene copy numbers of *nosZ*, were positively correlated to AMF root colonization measures (Fig.5c, Table S5). The ratio of *nosZ* copy numbers to the sum of *nirK* and *nirS* copy numbers (*nosZ*/(*nirK*+*nirS*)) was positively correlated to AMF root colonization measures (Table S5). All correlations were strongest with AMF vesicular root colonization (Fig.5b-d, Table S5). Correlations of *nirS* and the *16s rRNA* to AMF abundance were mostly absent (Table S5).

Most influential parameters affecting N_2O emissions

The multiple regression performed to identify the most influential parameters affecting N_2O emissions included microbial biomass C and N content and the abundance of *nirK* gene copy numbers. Overall, the model significantly (p=0.001) explained 58% of the variance in N_2O emissions (Table S4).

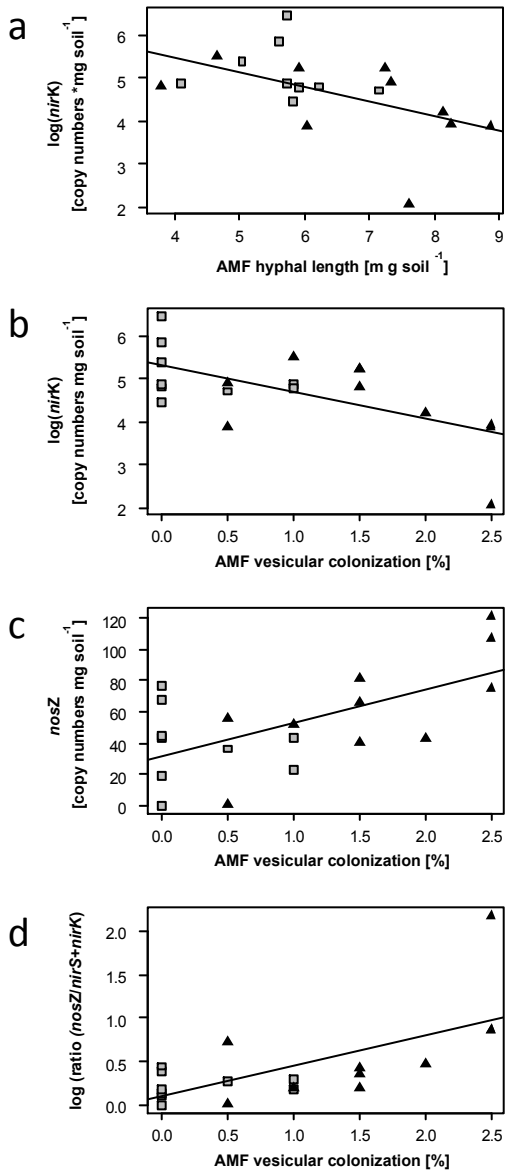

Figure 5: Pearson-correlations of AMF structures with denitrification gene copy numbers. Correlation of AMF extraradical hyphal length with gene copy numbers of *nirK* (log-transformed) (R^2=0.26, *P*=0.025) (a), and of AMF vesicular colonization with gene copy numbers of *nirK* (log-transformed) (R^2=0.39, *P*=0.004) (b), with gene copy numbers of *nosZ* (R^2=0.38, *P*=0.005)(c) and with the ratio *nosZ*(*nirK*+*nirS*) (log-transformed) (R^2=0.42, *P*=0.003). Correlations of other AMF parameters and denitrification genes are shown in Table S5. For all correlations the mean of three technical replicates per pot was used. Grey squares: tomato mutants (NM), black triangles: tomato wildtype (M).

Discussion

Soils are the major source of atmospheric N_2O. Still, the role of soil ecological interactions on denitrification and N_2O emissions are poorly understood and are only beginning to be revealed. While it is well established that AMF play a key role in ecosystems and provide a number of ecosystem services, it was unknown, until now, that AMF also influence N_2O emissions. Here, we demonstrate in two complementary experiments that AMF can contribute to reduced emissions of N_2O. As N_2O is a strong greenhouse gas and AMF are a very widespread group of organisms being distributed worldwide, the results suggest that AMF could play a role in the mitigation of climate change.

Our results point to several possible mechanisms by which the AMF symbiosis may reduce N_2O emissions. First, it is known that AMF can acquire significant amounts of nitrogen from soil (Bago et al., 1996; Govindarajulu et al., 2005; Johansen et al., 1993), suggesting that they can reduce substrate availability for denitrifying organisms. In the tomato-experiment, plant biomass and N contents were higher in the M treatment, while available soil NO_3^- was reduced. Consequently, one obvious mechanism by which AMF reduce N_2O emissions could be improved plant N nutrition resulting in a reduction of soil NO_3^- concentration, thus, limiting denitrification. However, in the grass-experiment, planted with a C3- grass known to show less pronounced responses to AMF (Hoeksema et al., 2010), plant biomass and N content, as well as available soil NO_3^- did not differ between treatments. This implies an additional involvement of mechanisms other than improved plant N nutrition to prevent N_2O emissions. The positive correlation of AMF extraradical hyphal length to soil microbial biomass N in this experiment suggests that increased N immobilization by the soil microbial biomass, also including AMF hyphae, may have contributed to reduced N_2O emissions in the grass-experiment.

Second, the availability of O_2 in soil is an important control of denitrification (Morley and Baggs, 2010) and is strongly correlated to soil water content (Smith, 1990). In the tomato-experiment, the WFPS declined faster in the M treatment during the gas measurements, probably due to enhanced plant transpiration induced by the higher plant biomass, or by enhanced water removal directly

induced by AMF (Auge, 2001; Khalvati et al., 2005; RuizLozano and Azcon, 1995). The faster water removal in the M treatment likely increased the oxygen availability in the soil and therefore reduced N_2O emissions, as denitrifying enzymes are expressed under low oxygen conditions to maintain respiration (Berks et al., 1995).

Third, in the tomato-experiment, we observed a significant negative correlation of AMF root colonization and extraradical hyphal length with *nirK*, a gene directly being involved in the production of N_2O, and a positive correlation of AMF root colonization with *nosZ*, a main gene consuming N_2O and reducing it to N_2. It has been shown that a relative reduction in denitrifying organisms containing the *nosZ* gene can lead to enhanced N_2O emissions (Philippot et al., 2011). There was a positive correlation of most AMF structures with the *nosZ*/(*nirS*+*nirK*) gene ratio (Table S5), indicating a relative increase in organisms containing *nos*Z with increased AMF abundance. Hence, these observations suggest that the presence of AMF is linked to changes in the denitrifier community composition. The absence of a relationship of AMF structures to *16S rRNA* implies that the total bacterial community size was not affected by the presence or absence of AMF, providing further support to our notion that AMF change the denitrifier community composition.

The increased CO_2 emissions in the M treatments confirm other studies (Cheng et al., 2012; Grimoldi et al., 2006; Nottingham et al., 2010) showing that AMF enhance CO_2 emissions from soil and suggest that C cycling was modified by AMF (Drigo et al., 2010). AMF induced shifts in C allocation into the soil can modify soil bacterial community composition (Toljander et al., 2007) and could also provide an explanation for the observed changes in the denitrifying communities as suggested by Veresoglou et al. (2012b). Moreover, AMF were reported to reduce C exudation from roots (Graham et al., 1981) and to exudate C from their hyphae (Hooker et al., 2007), suggesting that AMF enhance C transport into the bulk soil, where denitrifiers are less abundant and N_2 is the dominant denitrification endproduct (Cheneby et al., 2004.). Our observation that the abundance of the *nosZ* gene increased with AMF abundance supports this.

There is increasing evidence that many fungi are capable of denitrification and act as potentially significant sources of N_2O as they appear to lack a nitrous oxide reductase (Prendergast-Miller et al., 2011; Shoun et al., 1992). These studies have focused on ascomycete and basidiomycete species. Glomeromycota form a distinct linage (Schüssler et al., 2001) and direct assessment of any role in denitrification has not been performed. However, our results suggest that this group does not denitrify perhaps explaining why the AMF colonization is often reduced under waterlogged conditions (e.g. Ipsilantis and Sylvia, 2007; Mendoza et al., 2005).

In order to further understand which factors contributed to N_2O production, we performed a multiple regression. Our analysis revealed that microbial biomass C and N contents together with *nir*K abundance were the strongest predictors of N_2O emissions for the tomato-experiment, suggesting that the reduced N_2O emissions were caused by AMF-induced changes in soil microbial biomass and community composition.

In conclusion, the results presented here demonstrate that the AMF-symbiosis can reduce N_2O emissions from soil. Denitrification and related N_2O emissions are governed by complex interactions of various entangled factors. Also the effects the AMF symbiosis exerts on ecosystem processes are the result of complex interactions between fungus and plant. Disentangling these interactions and showing a direct cause-effect relationship is a challenging task that warrants further investigations. We show a hitherto unknown involvement of the AMF symbiosis in the reduction of N_2O emissions. Our results give a starting point for further investigations that should focus on the detailed mechanistic pathways by which the presence of AMF influences denitrifying communities and N_2O emissions.

The abundance of AMF in soil depends on soil nutrient availability and declines with fertilization and intensive land use (Egerton-Warburton and Allen, 2000; Helgason et al., 1998; Oehl et al., 2004). The results obtained here, suggest that a reduction of AMF abundance by intensive agricultural management and high fertilizer additions may initiate a cascade of below ground interactions that

further enhance N_2O emission from soil with potential negative consequences for the ozone layer and the earth's climate.

Acknowledgements

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Supporting Information

Supplementary methods

AMF inocula

Grass-experiment

Three AMF species, *Claroideoglomus claroideum* (previously named *Glomus claroideum*, isolate JJ132, (Jansa et al., 2002)), *Funneliformis mosseae* (previously named *Glomus mosseae* isolate BEG161, (Jansa et al., 2002)), and *G. intraradices* (isolate BEG 21, see van der Heijden et al. (2006) for description) were used in this experiment. They are common AMF in Swiss grasslands soils (Oehl et al., 2010). The fungi were propagated separately in the greenhouse in 3l pots, containing a 3:17 (v/v) soil:sand-mixture planted with *Plantago lanceolata*. The soil:sand-mixture had been inoculated with 5% inoculum of the respective fungal isolate. Every 2nd week, pots received 20 ml of a modified Hoagland solution (Hoagland and Arnon, 1950), containing one quarter of the original P concentration. A control inoculum, not containing AMF propagules, was produced under the same conditions. After three months of growth, pots were dried, emptied, and roots were cut into <5cm pieces and homogeneously mixed with the experimental substrate.

Tomato-experiment

The inoculum was a complex inoculum produced similarly to the inoculums used in the grass-experiment, with the exception that instead of specific AMF isolates, a similar amount of fresh field soil was added. The field soil used to produce the inoculum was the same soil used to fill the microcosms.

Soil chemical, physical, plant and microbial biomass analyses

Particle density, soil texture, organic C and soil P, available soil and leachate NO_3^- concentrations and soil pH were all analyzed using standard methods according to the reference methods of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL et al., 1996). Soil and leachate

NO_3^- contents were summed and used as a proxy for available soil NO_3^- at the end of the experiment. The particle density of the soil was determined to be able to calculate the WFPS in the microcosms as described in (Elliott et al., 1999) but using the actual particle density determined from our substrates. Dried shoots and roots were ground with a centrifuge mill (0.12mm), a dried soil subsample was milled in a ball mill and their total N content was determined with a FLASH Elemental Analyzer 1112 (Thermo Finnigan, Waltham, MA, USA). Plant shoot data was pooled for both harvests in the grass-experiment.

Microbial biomass C and N estimates by chloroform-fumigation-extraction (CFE) were carried out on duplicate samples according to Vance et al. (1987). Organic C (TOC) in the extracts was determined by infrared spectrometry after combustion at 850°C (DIMATOC® 2000, Dimatec, Essen, Germany). Total N was subsequently measured in the same sample by chemoluminescence (TNb, Dimatec, Essen, Germany). Microbial biomass C and N was calculated according to Jörgensen (1996) and Jörgensen and Mueller (1996).

AMF root colonization and hyphal length

The percentage of root length colonized by AM fungi was analyzed, after clearing the roots with 10% KOH and staining with a 5% Pen ink in vinegar mixture (Vierheilig et al., 1998), using a modified line-intersection method (McGonigle et al., 1990). 100 Intercepts were counted per sample.

The length of extraradical fungal hyphae in the soil was determined by a modified aqueous extraction and membrane-filter technique (Jakobsen et al., 1992) on triplicate soil samples of 10g in the grass-experiment and duplicate samples of 2g in the tomato-experiment. Hyphal length was calculated according to the modified Newman formula for calculating root length (Tennant, 1975).

Supporting Tables

Table S1: Results of the test for equal performance of the 2 tomato-genotypes in absence of AMF.

	tomato wildtype		**tomato BC1 -mutant**		
Cumulative gas emissions					
N_2O (µg N_2O-N kg $soil^{-1}$)	14.149	(±5.522)	12.064	(±9.675)	*P*=0.599
CO_2 (mg CO_2-C kg $soil^{-1}$)	1.677	(±0.440)	1.373	(±0.275)	*P*=0.448
Plant biomass					
Shoot (g kg $soil^{-1}$)	0.508	(±0.179)	0.461	(±0.116)	*P*=0.842
Root (g kg $soil^{-1}$)	0.065	(±0.013)	0.055	(±0.017)	*P*=0.640
Total (g kg $soil^{-1}$)	0.573	(±0.190)	0.516	(±0.132)	*P*=0.822
Plant N content					
Shoot (mg kg $soil^{-1}$)	0.022	(±0.008)	0.020	(±0.005)	*P*=0.827
Root (mg kg $soil^{-1}$)	0.001	(±0.000)	0.001	(±0.000)	*P*=0.587
Total (mg kg $soil^{-1}$)	0.024	(±0.008)	0.021	(±0.005)	*P*=0.815
Soil					
Soil N (g kg $soil^{-1}$)	1.088	(±0.316)	1.335	(±0.585)	*P*=0.346
Soil pH	7.758	(±0.042)	7.823	(±0.035)	*P*=0.294
Microbial biomass					
C content (mg kg $soil^{-1}$)	705.450	(±234.97)	892.924	(±234.66)	*P*=0.595
N content (mg kg $soil^{-1}$)	165.280	(±66.06)	229.397	(±73.75)	*P*=0.538
C/N ratio	6.769	(±1.63)	4.644	(±0.784)	*P*=0.318

The same field soil was used as in the main experiment. The soil was autoclaved for 90 min at 120°C two weeks before the start of the experiment. No additional AMF inoculum but a microbial wash was added to the pots for the test. The growth conditions and analyses were identical to the tomato-experiment. The test was performed independently from the main experiment and took place one month after the start of block 3 in the tomato-experiment. No significant difference in any of the measured variables could be detected. Values are means ± 1 SEM (n=5).

Table S2*:* Characteristics of the substrate mixture of the grass-experiment and the field soil plus inoculum used in the tomato-experiment.

Substrate characteristics	**Grass-Experiment**	**Tomato-Experiment**
Clay [%]	9.8	22.6
Silt [%]	14.5	30.9
Sand [%]	74.2	43.4
Humus [%]	1.5	3.1
pH (H_2O)	6.7	7.3
C_{org} [g kg $soil^{-1}$]	8.5	18.0
N total [g kg $soil^{-1}$]	7.6	28.0
available P [mg kg $soil^{-1}$]	0.19	0.99

Table S3: Primer pairs and reaction conditions used for gene copy number estimation of hyphal bag samples in the tomato-experiment.

Target	Primer	Sequence	Reaction conditions	Reference
Reference target	Mut-F	CCTACGGGAGGCAGGTC	95 °C, 15 min; 40 cycles: 95 °C for 10 s, 54 °C for 10 s, 72 °C for 10 s acquiring at 81 °C for 5 sec after each elongation	Daniell et al. (2012)
	Mut-R	ATTACCGCGGCTGCACC		
16S	Primer-1	CCTACGGGAGGCAGCAG	As above	Muyzer et al. (1993)
	Primer-2	ATTACCGCGGCTGCTGG		
*nir*K	876	ATYGGCGGVCAYGGCGA	95 °C for 15 min; 6 cycles of 95 °C for 10 sec, 63 °C for 10 sec, 72 °C for 10 sec; 40 cycles of 60 °C for 10 sec, 72 °C for 20 sec acquiring at 86 °C for 5 sec after each elongation	Hallin et al. (2009)
	1040	GCCTCGATCAGRTTRTGGTT		
*nir*S	Cd3aF	GTSAACGTSAAGGARACSGG	95 °C for 10 min; 40 cycles of 95 °C for 30 sec, 57 °C for 20 sec, 72 °C for 20 sec acquiring for 5 sec at 72 °C after each elongation	Michotey et al. (2000)
	R3cd	GASTTCGGRTGSGTCTTGA		Throback et al. (2004)
*nos*Z	nosZ2F	CGCRACGGCAASAAGGTSMSSGT	95 °C for 10 min then 40 cycles of 95 °C for 30 sec, 62 °C for 15 sec, 72 °C for 30 sec acquiring at 82 °C for 5 sec after each elongation.	Henry et al. (2006)
	nosZ2R	CAKRTGCAKSGCRTGGCAGAA		

Table S4: Results of a multiple regression for cumulative N_2O emissions in the tomato experiment to identify the most influential pathways, by which the presence of AMF affected N_2O emissions

Response: N_2O

	df	F-ratio	p-Value	Adjusted R^2
Regression	3	9.241	**0.001**	**0.579**
Residual	15			

Variable	Coefficient	SE	*t*-ratio	*P*-Value
(Intercept)	0.613	0.521	1.349	0.257
Microbial biomass C	0.002	0.001	3.507	**0.003**
Microbial biomass N	-0.017	0.004	-3.828	**0.002**
nirK	0.000	0.000	2.927	**0.010**

The multiple regression model included Block, rWFPS (% reduction in WFPS*h^{-1}), soil pH, available NO_3^-, Microbial biomass C and N content, total plant N content, denitrification gene copy numbers, *16s r-RNA* abundance and the ratio(*nosZ*/(*nirS*+*nirK*)). The minimal adequate model was obtained through stepwise deletion of terms. As no gene copy number data was available, no multiple regression was performed for the grass-experiment.

Table S5: Pearson correlations of AMF parameters with gene copy numbers of nirK (log-transformed), nirS (log-transformed), nosZ and 16s rRNA and the nosZ/(nirK+nirS)-ratio (log-transformed).

	log (*nirK*)			log (*nirS*)			*nosZ*			*16s rRNA*			log(ratio (*nosZ*/(*nirK*+*nirS*)))		
	r	R^2	*P*	*r*	R^2	*P*	*r*	R^2	*P*	*r*	R^2	*P*	*r*	R^2	*P*
TC [%]	-0.5	0.25	**0.026**	0.27	0.07	0.321	0.46	0.21	**0.042**	0.08	0.01	0.749	0.55	0.31	**0.011**
VC [%]	-0.61	0.38	**0.004**	0.51	0.26	**0.021**	0.61	0.38	**0.004**	0.35	0.12	0.130	0.64	0.42	**0.002**
AC [%]	-0.53	0.28	**0.017**	0.32	0.10	0.167	0.49	0.24	**0.027**	0.15	0.02	0.518	0.55	0.3	**0.012**
HL [m*g $soil^{-1}$]	-0.51	0.26	**0.022**	-0.03	0.00	0.900	0.11	0.01	0.635	0.08	0.01	0.731	0.39	0.15	0.093

For all correlations, the mean of three technical replicates per pot was used**.** Abbreviations: HC, hyphal colonization; VC, vesicular root colonization; AC, arbuscular root colonization; HL, extraradical hyphal length.

Significant correlations (*P*<0.05) are shown in bold.

Supporting Figures

Figure S1: Experimental microcosms in the grass-experiment. On the right, headspace is extended and closed for headspace gas sampling. A sleeve with a rubber seal and a removable cap was fit on the tubes to close the headspace airtight. The sleeve could be moved vertically along the tube surface to form the headspace chamber. For N_2O and CO_2 analyses, the cap contained two valves in which tubes for gas sampling could be inserted. A drain tap was inserted in the bottom of the tubes to allow leachate collection. For better drainage and filtering purposes, 1250g of an autoclaved sand-gravel mixture was added to the bottom of the tubes. The same system was used in the tomato-experiment.

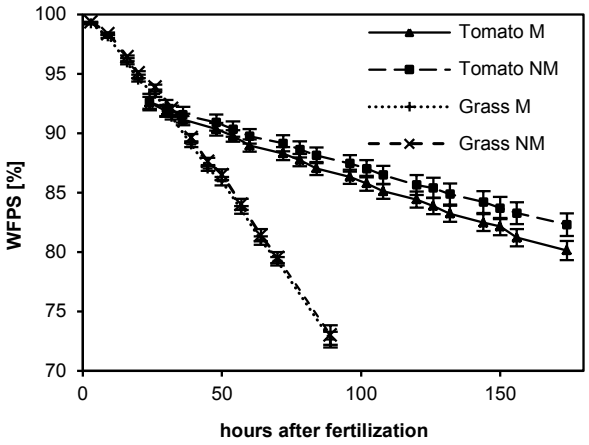

Figure S2: Soil water content expressed as WFPS during the gas sampling period in the grass- and tomato-experiment for the mycorrhizal (M) and the non-mycorrhizal (NM) treatment, respectively. Error bars represent ± 1 SEM (n=10 for the grass-experiment; for the tomato-experiment, n=9 for the NM, and n=10 for the M treatment). WFPS, water filled pore space.

Chapter 2

Mycorrhizal effects on nutrient cycling, nutrient leaching and N_2O production in experimental grassland

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Abstract

- Arbuscular mycorrhizal fungi (AMF) can enhance plant nutrition and growth. However, their contribution to nutrient cycling in ecosystems is still poorly understood. We hypothesize that AMF enhance the sustainability of plant-soil systems by reducing nutrient losses and enhancing plant nutrient uptake.

- We tested the AMF contribution to N and P cycling in experimental grassland microcosms including measurements of organic and inorganic leaching losses and N_2O fluxes with two different soil types and fertilized with different N forms (NO_3^- or NH_4^+).

- AMF reduced P leaching by 31%, enhanced plant P contents by 15% and increased P mobilization from soil by 18%. AMF reduced N_2O fluxes and NH_4^+ leaching in both soils. Leaching of dissolved organic N was reduced by 24% in the heath soil only. Plant N contents were increased by 13% in the pasture soil but not affected in the heath soil. The microbial biomass N content was higher with AMF.

- This is the first study providing a comprehensive assessment of the influence of AMF on N and P cycling, including effects on inorganic and organic nutrient leaching losses and N_2O emissions. We conclude that AMF can promote sustainable nutrient cycling but the effects on N cycling are context dependent.

Introduction

In agriculture, huge amounts of chemical fertilizers are applied to fields, of which around 50% remain unused by crops and are prone to getting lost from the system (Smil, 1999; Liu *et al.*, 2010). Nutrient losses are among the top environmental threats to ecosystems worldwide, as they can result in the pollution of waterways, harm the integrity of downstream ecosystems and add greenhouse gases to the atmosphere (Galloway *et al.*, 2003). Moreover, global phosphorus reserves used for the production of fertilizers are limited and may run out within decades (Gilbert, 2009) and the production of mineral N fertilizers is highly depended on declining fossil energy resources (Vance, 2001). Thus, there is an urgent need to increase nutrient use efficiency and reduce fertilizer application and nutrient losses in agro-ecosystems (Schlesinger, 2009). An increased nutrient use efficiency will help to maintain agricultural yields sufficient to feed a growing global population, and reduce environmental impacts .

Soil biota form an indispensable component of nutrient cycling. Several studies indicate that soil biota regulate nutrient transformations in soil and consequently determine plant nutrient availability. In addition to this, there is increasing evidence that soil biota influence the amount of nutrients being lost from soil via leaching or as gaseous forms (Plante, 2007; Robertson & Groffman, 2007; van der Heijden *et al.*, 2008; Philippot *et al.*, 2009; Wagg *et al.*, 2014). However, the role of specific groups of soil biota in regulating nutrient cycling is still poorly understood and the contribution of soil biota to several specific processes within the nutrient cycle (e.g. leaching of organic nutrients, denitrification and N_2O production) is unclear and has large uncertainties. Here we focus on the arbuscular mycorrhizal symbionts of plants.

Arbuscular mycorrhizal fungi (AMF) are a very widespread group of soil fungi, that form symbiotic relationships with the majority of land plants. It has been recognized that these fungi can improve plant growth by improving plant P nutrition (Sanders & Tinker, 1971; Clark & Zeto, 2000). AMF have also been shown to transfer N from soil to plants and in some (George *et al.*, 1995; Atul-Nayyar *et al.*, 2009; Cavagnaro *et al.*, 2012), but not all (Ames *et al.*, 1983; Hawkins *et al.*, 2000; Reynolds *et al.*,

2005) cases improve plant N nutrition. The relevance of AMF for plant N nutrition under ecological relevant conditions is still unclear (Fitter *et al.*, 2011).

While a substantial body of research focused on AMF effects on plant nutrition and performance, their involvement in other ecosystem processes, in particular in nutrient cycling has received relatively little attention (Rillig, 2004) and is not well understood. It was shown that AMF can reduce leaching losses of certain compounds of P and N , leading to the suggestion that AMF increase the nutrient use efficiency and sustainability of plant-soil systems (van der Heijden, 2010). However, only a limited number of studies has, so far, experimentally investigated the influence of AMF on nutrient leaching losses (Asghari *et al.*, 2005; van der Heijden, 2010; Asghari & Cavagnaro, 2011; Corkidi *et al.*, 2011; Asghari & Cavagnaro, 2012), mostly focusing on mineral leaching. Moreover, results obtained in these studies are based on a limited set of soil conditions, and none investigated all different forms of P and N potentially being prone to leaching. For example all but one study used substrates with sand contents ≥80%, while one study used an artificial growth substrate (Corkidi *et al.*, 2011). Moreover, all but one study determined leaching losses of inorganic N and P compounds only, while only one study (Asghari *et al.*, 2005) also determined total P losses but did not differentiate between different P fractions.

Apart from dissolved PO_4^{3-}-P, directly available to plants and therefore also defined as reactive P, P can also be leached in unreactive forms comprising all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates and particulate inorganic material, e.g. clays (Daniel & DeLaune, 2009).

These fractions can make up a substantial part of total leaching losses. For example, Ulen (1999) found up to 88%, while in a field study, Neumann *et al.* (2012) found fractions up to 60% of P leaching in unreactive forms. Non-mineral N leaching losses in dissolved organic form were found to make up to 64% by Dijkstra *et al.* (2007) , while Ghani *et al.* (2010) found losses up to 118 kg $ha^{-1}yr^{-1}$ in the form of dissolved organic N (DON), making up 97% of total N leaching loss. Because of the potentially important quantitative contribution of leaching losses in non-mineral form to total

leaching it remains unclear whether AMF can reduce overall N and P leaching losses, or only losses of certain nutrient compounds. To understand whether AMF contribute to improved nutrient recycling and enhanced sustainability, it is necessary to investigate the effects of AMF on nutrient leaching under a wider range of soil conditions and to assess all forms of N and P being leached.

Nitrogen can also be lost in gaseous forms. Estimates of N losses via denitrification, when nitrate is transformed to N_2O and N_2, are very variable and can range from 0 to more than 300 kg N ha^{-1} yr^{-1} lost from agricultural soils (Jambert *et al.*, 1997; Hofstra & Bouwman, 2005; Seitzinger *et al.*, 2006; van der Salm *et al.*, 2007). In earlier work we observed that AMF can reduce gaseous losses of N as N_2O (Bender *et al.*, 2013). The universality of this finding is still unclear and it is not known to which extent AMF influence N_2O emission under different soil conditions.

The relevance of AMF for N uptake and plant N nutrition is believed to be higher when N is provided in the form of NH_4^+ (Hamel, 2004; Govindarajulu *et al.*, 2005; Tanaka & Yano, 2005). If AMF N uptake is higher under NH_4^+ dominated conditions one would also expect stronger effects of AMF on N leaching under NH_4^+ dominated conditions, as AMF would reduce the availability of mineral soil N prone to leaching.

The aim of this study is to broaden our understanding of the influence of AMF on N and P cycling, testing AMF effects on plant nutrient uptake, nutrient mobilization, nutrient leaching and gaseous losses of N_2O.

We set up experimental grassland microcosms with two different soils (pasture soil and heath soil) and fertilized with different N forms (NO_3^- or NH_4^+). Because NH_4^+ is in most soils quickly transformed into NO_3^- through the process of nitrification, we chose a *Calluna vulgaris* dominated acid heath soil, which often have a low nitrification activity and in which NH_4^+ is the dominant N form (Troelstra *et al.*, 1990). We assessed the plant and soil nutrient pools, nutrient losses via leaching and fluxes of the greenhouse gas N_2O.

We hypothesized that

1) AMF reduce P losses through leaching. AMF reduce N losses, this effect is stronger under NH_4 fertilization, especially in the heath soil.

2) AMF affect N_2O emissions from denitrification. N_2O emissions are higher under NO_3^- fertilization and are negligible in the heath soil with NH_4 addition.

3) AMF improve plant P and N nutrition.

Material and methods

Experimental system

Grassland microcosms were established in PVC tubes with a diameter of 15 cm, a height of 40 cm, and a volume of approx. 7 L (see Fig. S1). A drain tap was inserted in the bottom of the tubes to allow leachate collection. A sleeve with a rubber seal and a removable cap was fit on the tubes to close the headspace airtight in order to collect gas samples to asses N_2O production. For N_2O measurements, the cap contained two valves in which tubes for gas sampling could be inserted. The sleeve could be moved vertically along the tube surface to form the headspace chamber. The microcosms were filled with 5.0 L of sterilized soil-sand-mixture (see below for details) containing 5.4% AMF inoculum and were planted with *Lolium multiflorum* var. Oryx, a common grass species in Swiss agricultural and natural grasslands often dominating temporary pastures in Switzerland (Nyfeler *et al.*, 2009). For better drainage and filtering purposes 1.3 kg of an autoclaved sand–gravel mixture was added to the bottom of the pots

The experimental setup comprised 2 soil types, 2 AMF treatments and 2 N fertilizer treatments (see below), each treatment combination being replicated 7 times, resulting in a total of 56 microcosms.

Soil, Inoculum and planting

The pasture soil was a *calcaric cambisol* collected from a long-term pasture site on an ecological farm near Research Station Agroscope ART in Zürich, Switzerland (47°43`11.83`` N, 8°53`65.25`` E). The pasture had been regularly manured.

The heath soil was a *dystric cambisol* collected from a dwarf shrub heath land dominated by *Calluna vulgaris and Vaccinium myrtillus* in the Black Forest, Germany (47°83`38.83`` N, 8°07`08.74`` E). Collected soils were 4 mm sieved, air dried and mixed with quartz sand to a soil:sand-ratio of 7:3 (v/v). This mixtures were then gamma irradiated with a maximum dose of 32 kGy to eliminate indigenous AMF. Four weeks after irradiation, the soil-sand mixtures were filled into the microcosms, moistened and incubated at room temperature for 2 weeks to allow stabilization of soil chemical properties before the experiment was initiated.

AMF inocula of *Funneliformis mosseae* (previously named *Glomus mossae*, isolate HG 505/ SAF 10), *Rhizophagus irregulare* (previously named *G. intraradices, isolate* SAF 22), and *Claroideoglomus claroideum* (previously named *G. claroideum*, isolate HG 181/ SAF 4), common AMF species in Swiss grassland and arable soils (Jansa *et al.*, 2002; Oehl *et al.*, 2010), were used in this experiment. All fungal isolates used can be found in the Swiss collection of AMF (SAF; www.agroscope.admin.ch/grandes-cultures-systemes-pastoraux/05911/07581/index.html). The fungal isolates had been propagated separately on *Plantago lanceolata* plants in 3 L pots containing a 3:17 (v/v) soil:sand-mixture in the greenhouse. A control inoculum not containing AMF propagules was produced under exactly the same conditions. Volumes of 270 ml of a mixture of the three AMF inocula or of control inoculum were mixed into the microcosms.

Soil irradiation not only eliminated indigenous AMF but also removed a significant proportion of other soil biota. Therefore, to include microbes from natural grassland and to allow a similar microbial background among the AMF and control inoculums, 100 ml of a microbial wash was mixed into the substrate for each microcosm (Koide & Li, 1989; van der Heijden *et al.*, 2006). The microbial wash was produced by suspending fresh field soil (either the pasture or the heath soil) and all used inocula in deionized water and subsequent filtering through a Schleicher and Schüll, No. 598 ½ filter paper (Schleicher & Schüll, Dassel, DE).

The characteristics of the final soil mixtures in the microcosms are summarized in Table 1. 270 ml of sterilized soil-sand mixture was added on top of the microcosms to reduce the risk of contamination between pots.

Before planting, seeds of *Lolium multiflorum* var. Oryx were surface sterilized by stirring in 1,25% bleach for ten minutes and rinsing with deionized water. They were allowed to germinate on 1.5% water agar for one week before planting 30 evenly spaced seedlings into the microcosms.

Table 1: Characteristics of the final soil-sand mixtures used to fill the microcosms.

	pasture soil	*heath soil*
pH	7.1	4.8
available P [mg*kg^{-1}]	1.08	1.12
mineral NO3-N [mg*kg-1]	1.96	1.40
mineral NH4-N [mg*kg-1]	0.70	2.26
N total [%]	0.12	0.15
chalk [%]	0.7	0
sand [%]	57.9	84.4
silt [%]	21.4	4.6
clay [%]	19.2	4.5
Corg [%]	0.87	3.77
humus [%]	1.5	6.5
C/N-ratio	7.4	24.6

Growth conditions

The microcosms were placed in a greenhouse with a 16h, 20°C day, and a 8h, 15°C night. Plants received natural light and supplemental illumination was provided by 400 W high-pressure sodium lamps to maintain a light level above 300 W m^{-2}. Pots were watered regularly by weight with deionized water to keep soil water content between 10 and 20%. Shoots were cut 5 cm above soil surface at 9 and 14 weeks after planting and were allowed to regrow.

Fertilization and Water Pulse

Weekly, each microcosm received 10 ml of a nutrient solution containing 1.5 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM Na_2MoO_4, 20 µM Fe-(Na)EDTA and either 9.98 mM KNO_3 for the NO_3^- fertilization or 4.99 mM

$(NH_4)_2SO_4$ for the NH_4^+ fertilization treatment, starting 8 weeks after planting. This corresponds to 0.77 kg N ha^{-1} and 0.26 kg P ha^{-1} or 1.4 mg N and 0.47 mg P per microcosm and fertilization event. After 18 weeks, the microcosms were watered to 90% water filled pore space (WFPS) with deionized water mixed with 10 ml of a nutrient solution (29.3 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$, 0.5 µM Na_2MoO_4, 20 µM Fe-(Na)EDTA and either 778 mM KNO_3 for the NO_3^- fertilization treatment or 389 mM $(NH_4)_2SO_4$ for the NH_4^+-fertilization treatment. This corresponded to a fertilizer pulse of 60 kg N ha^{-1} and 5 kg P ha^{-1} or 109 mg N and 9.1 mg P per microcosm for both fertilization treatments. The higher water and nutrient loadings were introduced to provide conditions conducive for nutrient leaching and denitrification and related N_2O emissions. Both soils are situated in regions with high annual rainfall (>1000 mm yr^{-1}) and, hence, commonly experience wet conditions as applied here.

N_2O flux measurements

N_2O fluxes were measured 24 h after fertilization and watering for 6 of the 7 replicates. For the N_2O measurements, the headspace was adjusted to a height of 20 cm above soil surface (4 L volume) and closed for a period of 10 minutes with the headspace gas pumped through a TEI 46c automated N_2O analyser (Thermo Fisher Scientific, Waltham, USA).

Watering, fertilization, and gas measurements proceeded in 15 minute steps, so that the time between fertilization and N_2O measurement was the same for all microcosms. The cap used to close the headspace was non-transparent.

Artificial rain and harvest

After the N_2O measurements, the microcosms were exposed to a simulated rainfall of 1.5 L with a rain simulator as described in Knacker *et al.* (2004). The drain tap in the bottom of the pots was opened and leachate was collected. After approximately 2 hours, when no more leachate dripped out of the microcosms, leachate was weighed and a subsample was taken for nutrient analysis. Shoots where cut at soil surface, dried at 60°C and weighed. The substrate was removed from the microcosms and all visible roots where collected, rinsed with water and a weighed subsample was

taken and stored in 50% Ethanol. Remaining roots were dried and weighed. The soil was mixed thoroughly and samples were taken for soil and microbial analyses.

Nutrient analyses

Leachates

The leachates, which passed through the sand-gravel mixture at the bottom of the microcosms, were very clear and were not additionally filtered before analyses. Leachates were chemically analysed for nutrient concentrations. NO_3^--N, NO_2^--N and dissolved PO_4^{3-}-P were determined using a Dionex DX500 anion chromatograph (Dionex Corporation, Sunnyvale, CA). Total P in leachate was determined using Oxisolv® (Merck, Darmstadt, DE) oxidation prior to the photometric analysis with a spectrophotometer (Helios Gamma, Thermo Scientific, Digitana AG, Switzerland) using the molybdenum blue ascorbic acid method (Watanabe & Olsen, 1965). NH_4^+-N was analysed using a Skalar segmented flow analyser (Skalar, Breda, NL) according to the reference methods of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL *et al.*, 1996). Total dissolved N (TDN) was measured by chemoluminescence (DIMATOC® 2000 coupled with a DIMA-N analyser, Dimatec, Essen, DE). For PO_4^{3-}-P, NO_3^--N, NO_2^--N and NH_4^+-N, 46 %, 25 %, 37% and 27 % of the samples yielded concentrations below the detection limit, respectively.

The measured nutrient concentrations were multiplied with the leachate volume to get the total amount lost per microcosm. Amounts of NO_2^--N were low and were added to the NO_3^--N values. Dissolved organic N (DON) was calculated by subtracting the amounts of mineral N (NO_3^--N and NH_4^+-N) from TDN. In cases where no mineral N leaching was detected (7 % of the samples), the complete amount of TDN leached was assumed to be in dissolved organic form.

The amount of PO_4^{3-}-P in the samples was labelled reactive P. The difference between total P and reactive P was labelled unreactive P. This fraction comprises all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates and particulate inorganic material, e.g. clays (Daniel & DeLaune, 2009). In cases where no reactive P leaching was detected, the complete amount of total P leached was assumed to be in unreactive form.

Plant and Soil

Dried shoot and root samples were ground with a centrifuge mill (0.12 mm) and a dried soil subsample was milled in a ball mill. All shoot harvests were pooled and N concentrations of the shoot samples, roots and soils were determined with a FLASH Elemental Analyser 1112 (Thermo Finnigan, Waltham, MA, USA). Shoot and root P concentrations were determined photometrically using the molybdenum blue ascorbic acid method (Watanabe & Olsen, 1965) after dry ashing.

Soil texture, particle density, organic C, $CaCO_3$ and soil pH, available soil P extracted with CO_2-saturated water and mineral soil N (NO_3^--N and NH_4^+-N), extracted with 0.0125 M $CaCl_2$ were all analysed using standard methods according to the reference methods of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL *et al.*, 1996). For soil mineral NO_3^--N and soil mineral NH_4^+-N, 39 % and 29 % of the data points were below the detection limit, respectively. The particle density of the soil was determined to be able to calculate the WFPS in the microcosms as described in Elliott *et al.* (1999) but using the actual particle density determined from our substrates.

Soil microbial biomass C and N

Soil Microbial Biomass C and N was determined by Chloroform Fumigation Extraction (Vance *et al.*, 1987) in duplicate on 20 g (dry matter) subsamples extracted with 80 ml of a 0.5 M K_2SO_4 solution. Organic C (TOC) was determined by infrared spectrometry after combustion at 850°C (DIMATOC® 2000, Dimatec, Essen, Germany). Total N was subsequently measured in the same sample by chemoluminescence (TNb, Dimatec, Essen, Germany). Microbial biomass C and N was calculated according to Jörgensen (1996) and Jörgensen and Mueller (1996). These measurements also comprise the C and N contents of AMF hyphae as these structures are also decomposed by the chloroform treatment (Olsson *et al.*, 1995).

AMF root colonization

The percentage of root length colonized by AMF was determined from root samples stored in 50% ethanol after staining with pen ink (Vierheilig *et al.*, 1998) and using a modified line-intersection method for 100 intersections (McGonigle *et al.*, 1990).

Statistical analyses

All leachate, plant and soil data were analysed using ANOVA with Soil-type, N fertilizer and AMF treatment as factors and all interactions. To account for the blocking design of the experiment, the Block effect was added first in the model. In case of significant interactions, means were compared using Tukey`s HSD test. ANOVA assumptions were controlled by plotting residuals against fitted values. Some nutrient concentration variables contained high numbers of values below the detection limit resulting in not available data points. For variables with more than 30% of the values being not available, no ANOVA was performed.

Pearson correlations were performed to test for linear relationships between plant N and P contents and N and P mobilized from soil during the experiment. All statistical analyses were performed using the R statistical software, Version 2.14.1.

Results

Leaching losses

Leaching of total P was overall reduced by 31% in presence of AMF (Table 2, Fig. 1a). Leaching of unreactive P made up the biggest fraction of total P leaching (approx. 64 % and 90 % for the pasture and heath soil, respectively) and was overall reduced by AMF (24 % reduction) (Table 2 , Fig. 1b). Leaching losses of reactive P were low.. In the pasture soil no differences between the treatments could be observed. In the heath soil, only 6 of 28 samples yielded detectable reactive P concentrations, exclusively being derived from the NM treatments (Fig. 1c).

There was on average a 69% reduction of NH_4^+ leaching by AMF, irrespective of soil type and N fertilizer (Table 2, Fig. 2a). Leaching losses of NO_3^- were higher in the heath soil and with NO_3^- fertilization and were not affected by AMF (Table 2, Fig. 2b). In the heath soil fertilized with NH_4^+, no NO_3^- could be detected in the leachates (Fig. 2b).

There was a significant interaction of AMF with soil type on DON leaching (Table 2). In the heath soil, DON leaching loss was on average 24 % lower in the AMF treatment (Tukey HSD, P=0.006), while in

the pasture soil, there was no significant effect of AMF (Tukey HSD, *P*=0.87) (Fig. 2c). Leaching of DON was higher in the heath soil and did not respond to the type of N fertilizer (Fig. 2c).

TDN leaching was overall not affected by AMF and was higher with NO_3^- than with NH_4^+ fertilization. The highest TDN leaching was observed in the heath soil receiving NO_3^- fertilizer resulting in a significant interaction of soil type with N fertilizer (Tables 2 and S1).

Table 2: F-values and significance levels of 4-way ANOVAS analyzing the effects of Soil-type, N-fertilizer and AMF and all interactions on the different nutrient compounds in leachate, Plant biomass and nutrient concentrations, soil nutrient pools, Microbial biomass C and N contents and N_2O fluxes. NO_3^--N and NH_4^+-N leaching data and soil mineral NH_4^+-N data were below the detection limit for some of the samples and N_2O fluxes were measured for 6 replicates only, resulting in reduced residual degrees of freedoms. Significance levels are indicated by asteriscs (***, *P*<0.001;**, *P* <0.01; *, *P*<0.05; ·, P<0.1)

	Source of variation								
Response									
Leaching	Block	Soil	N-fertilizer	AMF	Soil x N-fertilizer	Soil x AMF	N-fertilizer x AMF	Soil x N-fertilizer x AMF	Residuals
df	6	1	1	1	1	1	1	1	42
unreactive P[a]	**4.81** ***	**122.56** ***	0.20	**11.72** **	0.24	0.23	0.16	0.43	
Total P[a]	**2.95** *	**30.40** ***	0.80	**13.07** ***	0.48	2.08	0.59	0.03	
TDN	**6.99** ***	**123.96** ***	**149.79****	1.29	**47.22** ***	1.49	0.01	0.12	
DON	**2.52** *	**110.38** ***	1.09	3.74.	2.75	**9.03** **	1.75	0.35	
df	6	1	1	1	1	1	1	1	30
NO_3^--N	**7.25** ***	**199.98****	**34.27** ***	0.27	-	0.95	0.43	-	
df	6	1	1	1	1	1	1	1	127
NH_4^+-N	**3.68** **	0.50	2.52	**8.76** **	0.10	0.19	2.64	0.44	
N_2O flux									
df	6	1	1	1	1	1	1	1	34
N_2O-flux[b]	1.27	0.14	**202.07****	**13.10** ***	**8.86** **	3.31.	**7.82****	0.92	
Plant parameters									
df	6	1	1	1	1	1	1	1	42
total biomass	**4.84** ***	**5.09** *	0.02	2.17	0.20	**9.50** **	0.00	0.59	
total plant P	**2.84** *	**7.60** **	0.91	**11.01** **	0.36	2.61	2.16	0.04	
total plant N	1.53	**305.80** ***	0.00	1.61	0.59	**10.03** **	1.65	1.34	
plant N:P ratio	1.85	**15.67** ***	0.02	**9.08** **	0.41	0.50	1.46	0.05	
Soil									
Soil N	1.49	3.14	1.10	0.13	**5.70** *	0.12	0.39	3.79	
available soil P	0.92	**4.81** *	0.07	0.00	2.60	0.10	3.63.	0.09	
MiBi C	1.63	**6.66** *	1.12	**16.73** ***	3.99.	0.45	0.64	0.58	
MiBi N	1.80	**96.24** ***	1.12	**17.04** ***	2.06	3.50.	1.21	1.82	
MiBi C/N	**5.03** ***	**346.74** ***	0.66	0.00	0.28	2.47	0.08	1.49	
df	6	1	1	1	1	1	1	1	26
min NH_4^+-N[a]	1.49	**12.26** **	**48.42****	1.70	**32.86** ***	0.17	1.41	0.11	

[a] log-transformed; [b]squareroot-transformed

N_2O fluxes

In both soils, N_2O fluxes 24 h after fertilization were significantly reduced by AMF by 47.1% when NO_3^- fertilizer was added (Tukey HSD, *P*=0.004). When NH_4^+ fertilizer was added, N_2O fluxes were much lower and not affected by AMF (Tukey HSD, *P*=0.937) (Fig. 3). This was reflected by a significant interaction of AMF with N fertilizer type (Table 2).

Plant biomass and nutrient contents

There was a significant interaction between the effects of soil type and AMF on plant biomass (Table 2). While in the pasture soil, plant biomass was increased by 22% with AMF (Tukey HSD, *P*=0.013) there was no effect of AMF on plant biomass in the heath soil (Tukey HSD, *P*=0.67) (Fig. 4a). Plant P contents were, averaged across both soil types and fertilizer treatments, significantly increased (+15%) with AMF and were higher in pasture soil than in heath soil (Fig. 4b, Table 2). Total Plant N contents were also higher in the pasture than in the heath soil (Fig. 4c). There was a significant interaction of soil type with AMF on plant N contents (Table 2). In the pasture soil, Plant N was increased by 13% with AMF (Tukey HSD, *P*=0.016), while in the heath soil, plant N did not differ between the AMF treatments (Tukey HSD, *P*= 0.54). The plant N:P ratio was significantly higher in the pasture than in the heath soil and was constantly reduced by 18% with AMF (Tables 2 and S1).

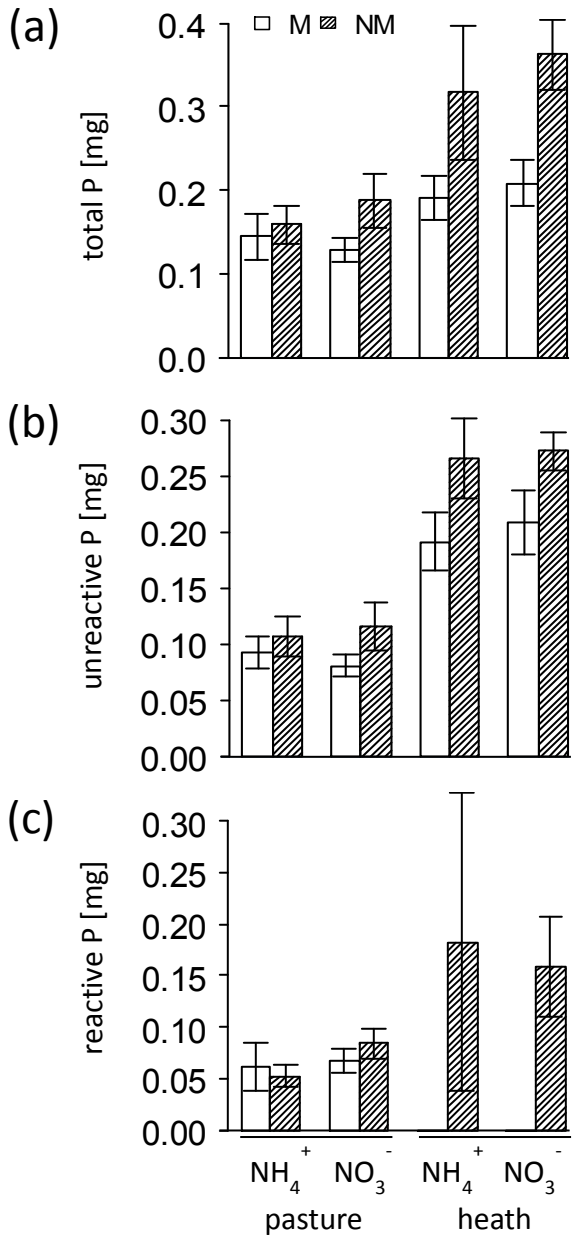

Figure 1 : Leaching losses of total (a), unreactive (b) and reactive (PO_4^{3-}) (c) P from grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars). Error bars indicate ±1 SE (n=7, for unreactive P, n is partially lower because several data points were below the detection limit).

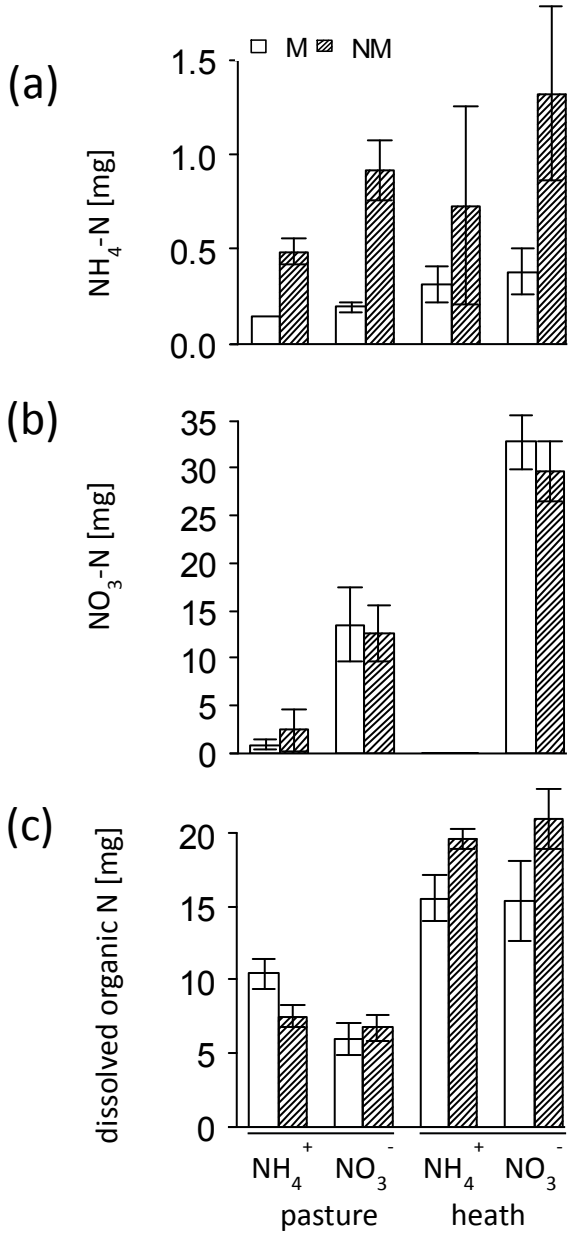

Figure 2: Leaching losses of NH_4^+-N (a), NO_3^--N (b) and dissolved organic N (b) from grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars). Error bars indicate ±1 SE (n=7, for NH_4^+-N, n is partially lower because several data points were below the detection limit, in the heath soil fertilized with NH_4^+, no NO_3^--N was detected in the leachates).

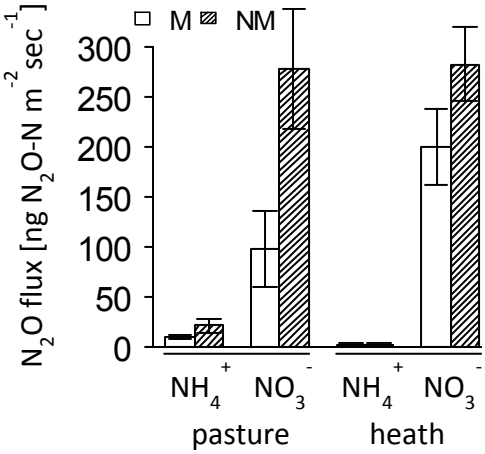

Figure 3: N_2O fluxes in grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars) measured 24 h after the N fertilizer had been applied. Error bars indicate ±1 SE (n=6).

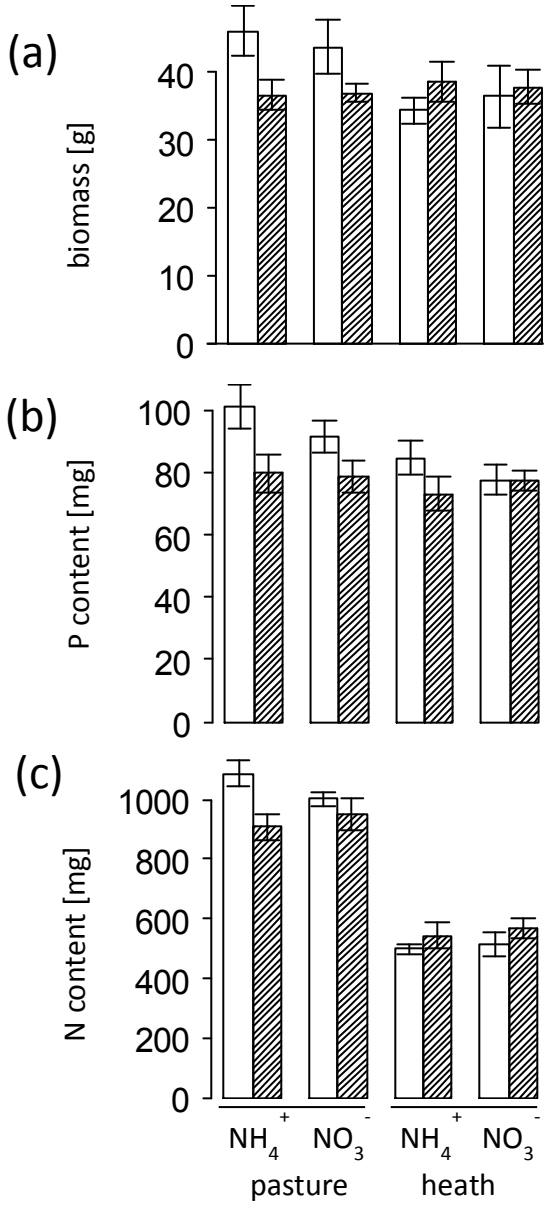

Figure 4: Plant biomass (a) and plant P (b) and N contents (c) in grassland microcosms filled with two different soil types (pasture and heath) combined with two N fertilizers (NH_4^+ and NO_3^-) and either inoculated with AMF (M, blank bars) or receiving a non-mycorrhizal control inoculum (NM, shaded bars). Error bars indicate ±1 SE (n=7).

Soil nutrient contents

Available soil P at the end of the experiment was significantly higher in the pasture soil compared to the heath soil (Tables 2 and S1). However, there was no effect of N fertilizer or AMF on available soil P (Tables 2 and S1).

Soil mineral NO_3^- -N contents were of similar magnitude with both fertilizer types and AMF treatments in the pasture soil. In the heath soil fertilized with NH_4^+, soil mineral NO_3^--N contents were slightly lower as in the pasture soil and were also not affected by AMF. In the heath soil fertilized with NH_4^+, no soil mineral NO_3^--N could be detected (Table S1).

For soil mineral NH_4^+-N contents, the ANOVA revealed a significant interaction of soil type with N fertilizer (Table 2). In the pasture soil with both fertilizers and in the heath soil fertilized with NO_3^-, soil mineral NH_4^+ contents were of similar magnitude (Tukey HSD, P>0.05). In the heath soil fertilized with NH_4^+, mineral NH_4^+-N contents were distinctly higher compared to the other soil-fertilizer combinations (Tukey HSD<0.001) (Table S1).

AMF root colonization and Microbial biomass

AMF root colonization was higher in the pasture soil than in the heath soil.

Root length colonized by AMF was 57.3 and 12.4% for the pasture and the heath soil, respectively. No AMF colonization was detected in the NM treatments (Table S1).

Microbial biomass C and N contents were overall significantly increased by 19% in the M compared to the NM treatments (Table 2) and were both higher in the pasture soil compared to the heath soil (Tables 2 and S1). The microbial biomass C:N ratio was significantly increased in the heath soil compared to the pasture soil (Tables 2 and S1).

P and N mass balance

We compared the initially available amounts of P and N in soil plus the amounts added with fertilization, with the respective amounts at the end of the experiment and amounts removed by plants, leachate and N immobilized in microbial biomass in Tables 3 and 4.

Of the P initially available in soil and added with fertilizer, between 0.67 and 1.92% had been lost through leaching. About 3.7 times the amount of initially available and fertilized P was found in the plant soil system at the end of the experiment (Table 3). This indicates that most P detected at the end had been mobilized from initially non-available soil P resources. On average 17.5% more P had additionally been mobilized from initially non-available soil P resources in the presence of AMF ($F_{1,42}$=9.81; P=0.003)(Table 3).

Of the mineral N initially available in soil and added with fertilizer, between 5.7 and 29.8% had been lost through leaching. About 4.7 times the amount of mineral N initially present and N fertilized was found in the plant soil system at the end of the experiment (Table 4). This indicates that most N detected had been mobilized from initially organic soil N resources. On average, 17.3% more N had

additionally been mobilized from organic soil N resources in the presence of AMF in the pasture soil (AMF:soil interaction: $F_{1,42}$=11.63; *P*=0.001; Tukey HSD, *P*<0.001), while the amount of additionally mobilized N was slightly lower (-4.8%) in presence of AMF in the heath soil (Tukey HSD, *P*= 0.9)(Table 4).

Table 3: Phosphorus-Budget of grassland microcosms filled with two different soil types combined with two N fertilizers and either inoculated with AMF or receiving a non-mycorrhizal control inoculum and the relative change of the respective P pools in the M treatment compared to the NM treatment. Positive signs indicate a relative increase of the respective nutrient pool in the M treatment compared to the NM treatment, a negative sign indicates a relative decrease in the respective nutrient pool in the M treatment.

	Pasture soil								Heath soil							
	NH_4- fertilizer				NO_3-fertilizer				NH_4- fertilizer				NO_3-fertilizer			
	M		NM		M		NM		M		NM		M		NM	
	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
Available P in soil initially	7.3		7.3		7.3		7.3		7.0		7.0		7.0		7.0	
P fertilized	11.9		11.9		11.9		11.9		11.9		11.9		11.9		11.9	
sum	19.1	100	19.1	100	19.1	100	19.1	100	18.9	100	18.9	100	18.9	100	18.9	100
P taken up by plants	101.0	527.8	79.8	416.7	91.4	477.3	78.8	411.5	84.7	448.4	73.1	387.0	77.7	411.4	77.6	410.9
P loss in leachate	0.1	0.76	0.2	0.83	0.1	0.67	0.2	0.98	0.2	1.01	0.3	1.68	0.2	1.10	0.4	1.92
Available P remaining in soil	6.9	36.2	6.2	32.6	5.6	29.4	6.5	34.2	5.5	29.3	4.8	25.5	5.6	29.8	6.1	32.3
P additionally mobilized	89.0	464.8	67.0	350.1	78.0	407.4	66.4	346.7	71.5	378.7	59.3	314.1	64.6	342.3	65.2	345.2
	% change induced by AMF															
P taken up by plants	+26.7				+16.0				+15.9				+0.1			
P loss in leachate	-8.9				-31.7				-39.7				-42.6			
Available P remaining in soil	+11.3				-14.2				+15.0				-7.7			
P additionally mobilized	+32.8				+17.5				+20.6				-0.8			

Table 4: Nitrogen-Budget of grassland microcosms filled with two different soil types combined with two N fertilizers and either inoculated with AMF or receiving a non-mycorrhizal control inoculum and the relative change of the respective P pools in the M treatment compared to the NM treatment.

Positive signs indicate a relative increase of the respective nutrient pool in the M treatment compared to the NM treatment, a negative sign indicates a relative decrease in the respective nutrient pool in the M treatment.

	Pasture soil								Heath soil							
	NH_4- fertilizer				NO_3-fertilizer				NH_4- fertilizer				NO_3-fertilizer			
	M		NM		M		NM		M		NM		M		NM	
	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
Nmin in soil initially	63.9		63.9		63.9		63.9		57.0		57.0		57.0		57.0	
N fertilized	117.3		117.3		117.3		117.3		117.3		117.3		117.3		117.3	
sum	181.2	100	181.2	100	181.2	100	181.2	100	174.3	100	174.3	100	174.3	100	174.3	100
N taken up by plants	1088	600.2	908	501.0	1003	553.5	951	524.8	498	285.8	544	312.3	514	295.0	567	325.3
N loss in leachate	11.2	6.2	10.3	5.7	19.5	10.8	20.1	11.1	15.8	9.1	20.2	11.6	48.4	27.8	52.0	29.8
Microbial biomassN	298	164.4	220	121.6	272	150.1	239	132.1	165	94.8	147	84.2	190	109.2	167	96.0
Nmin remaining in soil	15.8	8.7	14.8	8.2	14.5	8.0	13.2	7.3	28.8	16.5	19.6	11.2	13.6	7.8	14.7	8.4
N additionally mobilized	1231	679.5	972	536.5	1128	622.4	1043	575.3	533	306.1	556	319.3	592	339.8	627	359.6
	% change by AMF															
N taken up by plants	+19.8				+5.5				-8.5				-9.3			
N loss in leachate	+8.6				-3.1				-21.8				-6.8			
Microbial biomassN	+35.2				+13.6				+12.6				+13.7			
Nmin remaining in soil	+6.5				+10.2				+47.0				-7.3			
N additionally mobilized	+26.6				+8.2				-4.1				-5.5			

Discussion

This is the first study providing a comprehensive assessment of the influence of AMF on N and P cycling, including effects on leaching losses and N_2O emissions. We show for the first time that AMF are capable to reduce the leaching of dissolved organic N and unreactive P compounds.

Other studies showed that AMF can reduce reactive P leaching losses, but a contribution of AMF to the reduction of unreactive P leaching had so far not been shown. This finding is important, because in our study and other studies investigating P leaching (Ulen, 1999; Neumann *et al.*, 2012), a considerable fraction of total P leaching occurred in unreactive forms. A reduction of reactive P leaching by AMF can be explained by enhanced uptake of P from soil solution due to exploitation of a bigger soil volume by AMF rooting systems compared to non-mycorrhizal plant roots (Jakobsen *et al.*, 1992; Jansa *et al.*, 2005). A reduction of unreactive P leaching can be related to an either direct, or

indirect increase of mineralization of organic P compounds in the presence of AMF and subsequent uptake of the inorganic products (Jayachandran *et al.*, 1992). Also, the utilization of insoluble inorganic P compounds by AMF has been shown (Bolan *et al.*, 1987) and probably contributed to the reduction of unreactive P leaching.

In the presence of AMF, the mobilization of initially non-available soil P resources was significantly enhanced compared to the non-mycorrhizal treatments indicating an overall increase in P cycling by AMF. As the mobilization of P from soil resources was strongly correlated to plant P contents, this effect is likely to be indirectly caused via AMF mediated improvements in plant nutrition.

Total P leaching losses depended strongly on soil type and were much higher in the heath soil compared to the pasture soil. The heath soil had a much higher sand and organic matter content than the pasture soil (Table 1), both being properties often reducing P sorption capacity of soils (Weaver *et al.*, 1988; Atalay, 2001; Daly *et al.*, 2001). The calcareous pasture soil, hence, probably had a higher ability to fix P and, consequently, P leaching losses were lower.

AMF have been shown to enhance the mineralization of organic matter (Hodge *et al.*, 2001; Feng *et al.*, 2003; Atul-Nayyar *et al.*, 2009; Cheng *et al.*, 2012), as well as to directly take up organic N compounds (Hawkins *et al.*, 2000; Whiteside *et al.*, 2009; Whiteside *et al.*, 2012) from soil. Hence, the reduction in DON leaching in the heath soil could result from enhanced DON mineralization, resulting in mineral N release, or from direct uptake of DON in the presence of AMF.

It is well established that AMF can take up NH_4^+ from soil and transfer it to their host plants (Frey & Schuepp, 1993; Johansen *et al.*, 1993; Mäder *et al.*, 2000). Moreover, it has been shown that AMF preferentially take up NH_4^+ rather than NO_3^- (Govindarajulu *et al.*, 2005; Tanaka & Yano, 2005). Hence, the observed overall reduction in NH_4^+ leaching through AMF could result from enhanced NH_4^+ immobilization from the soil by AMF rooting systems. This would reduce the amount of NH_4^+ in the soil prone to leaching.

In the acid heath soil fertilized with NH_4^+, we successfully created NH_4^+ dominated conditions as indicated by the absence of NO_3^- in the soil and leachates (Fig. 2b and Table S1). Under these

conditions, we expected the strongest reduction of N leaching losses by AMF as plants were suggested to rely more on AMF for N-acquisition when NH_4^+ is the dominating N form (Johansen *et al.*, 1993; Hamel, 2004). In contrast to our expectations, we did not observe pronounced effects of AMF on NH_4^+ leaching losses and also plant N contents were not enhanced in that treatment. The high amounts of mineral NH_4^+ in the soil at the end of the experiment (Table S1) either indicate that NH_4^+ was not the preferred form for biological N uptake under these conditions or that NH_4^+ uptake and translocation by AMF is a slow process (e.g. the majority of NH_4^+ fertilizer (88.6%) was applied, 1 day before harvesting the experiment).

Microbial biomass N contents were constantly higher in the M treatments. Earlier work showed that AMF can immobilize substantial amounts of N in their hyphal biomass (Hodge & Fitter, 2010). AMF hyphae could have served as a N sink in the M treatments, hence, reducing N leaching. Another possibility is, that the presence of AMF promoted microbial communities more efficiently immobilizing N.

In the pasture soil, plant biomass and N contents were increased by AMF, while they tended to be reduced in the heath soil. Increased plant N uptake in the M treatments might, thus, have contributed to the significantly reduced NH_4^+ leaching in the pasture soil. Effects on leaching losses in the heath soil can, however, not be explained by increased plant N uptake.

The strongly reduced root colonization by AMF in the heath soil compared to the pasture soil is in line with the literature reporting reduced AMF root colonization under low pH conditions (van Aarle *et al.*, 2002; Goransson *et al.*, 2008). Several of the observed AMF effects in this study were, however, more pronounced in the acid heath soil. This indicates, that even when AMF abundance in the roots is low, they can still exert a significant influence on nutrient cycling processes.

Plant N:P ratios have been proposed as an indicator of nutrient limitation for plants (Koerselman & Meuleman, 1996). The biomass N:P ratios in this experiment were all below 14, indicating N limited conditions. However, N limitation seems to have been more severe in the heath soil with an average plant N:P ratio of 6.9 compared to an average plant N:P ratio of 11.5 in the pasture soil. It has been

proposed that nutrient stoichiometry can determine whether the AMF symbiosis turns out to be mutualistic or antagonistic (Johnson, 2010). In line with this, AMF had a stronger impact on plant biomass in the pasture soil which had a lower relative P-availability compared to the heath soil. Thus, the differences in the AMF effects on plant growth between the soil types could result from difference in nutrient availability and nutrient ratios between the soils.

Furthermore, both soils strongly differed in their C:N ratios, the heath soil showing a C:N ratio of 24.6 which is remarkably higher than the C:N ratio of 7.4 found for the pasture soil.

N availability has been reported to be reduced and competition between plants and the soil microbial community for N to be enhanced when the C:N ratio is high. The competitive ability of the soil microbial community to acquire N is believed to be superior to plants under these conditions (Kaye & Hart, 1997).

Microbial biomass N content overall was higher with AMF, while in the pasture soil, plant N contents were also increased. In the heath soil, plant N contents were much lower and they were still slightly lower in the presence of AMF. These result suggest, that the microbial biomass was overall capable to improve its` N nutrition in the presence of AMF. Under conditions of relatively high N availability in the pasture soil, plant N nutrition also benefitted from the presence of AMF. In the heath soil, with a relatively lower N availability, plants did not benefit from AMF in terms of N nutrition, indicating a stronger competitive ability for N of the microbial biomass under these conditions. This agrees with the assumption that AMF contribute to plant N supply only under conditions where N is available in amounts sufficient to satisfy AMF demands and additionally allow AMF N transfer to the plant hosts (Fitter *et al.*, 2011).

This study confirms our earlier work, that AMF reduce N_2O emissions from soil (Bender *et al.*, 2013). As NO_3^- is the main substrate for producing N_2O, it is to be expected that we observed higher N_2O emissions under NO_3^- fertilization and negligible emissions in the heath soil fertilized with NH_4^+ where no NO_3^- was detected (Table S1). Note that, the reduction of N_2O fluxes measured 24 h after fertilization can only give an indication of whether AMF reduced N_2O emissions, as these data

represent a snapshot of N_2O emissions in time and do not allow inferences about total N_2O losses associated with the amount of fertilizer applied. Still, the significantly reduced N_2O fluxes over both soil types provide, in addition to two further experiments (see Bender *et al.*, 2013), a strong indication that AMF affect N_2O emissions under a wide range of conditions, including acidic soils fertilized with NO_3^-.

Conclusions

The results presented here comprehensively show the influence of AMF on N and P cycling, including effects on leaching losses and N_2O emissions. We provide a first evidence that AMF are capable of reducing the leaching of dissolved organic N and unreactive P compounds. This study strikes the importance of AMF on efficient nutrient cycling. While AMF reduced P losses through leaching, they simultaneously increased the amount of available P being cycled through our model grasslands. These results clearly show, that AMF can increase the P use-efficiency on an ecosystem level, by enhancing nutrient mobilization while reducing losses.

For N, the results were more complex. In the pasture soil, plant N contents, microbial biomass N and the total amount of mineral N cycled through the grassland system was significantly enhanced by AMF, but total N leaching losses were not affected.

In the heath soil, however, AMF did not enhance the amount of cycled N, and the presence of AMF had no significant effect on plant N contents. However, there was a partly reduction in N leaching losses by AMF under these conditions. Taken together these results indicate that a potential reduction of N leaching is uncoupled from effects on plant N uptake and that the interference of AMF with the N cycle is context dependent.

A huge fraction of N and P losses occurred in dissolved organic or unreactive forms, especially for P. Leaching of organic and unreactive compounds were reported to comprise significant fractions of total leaching losses in several ecosystems (Schoenau & Bettany, 1987; Ulen, 1999; Smolander *et al.*, 2001; Ghani *et al.*, 2010). Thus, our results imply that leaching of organic and unreactive compounds must not be ignored when using AMF effects on nutrient leaching to proclaim increased nutrient

retention and sustainability though AMF. It has been shown that soil biodiversity and soil community composition are key drivers for the functioning of ecosystems (Wagg *et al.*, 2014). Our results show that these beneficial effects of soil communities might, in huge parts, have to be attributed to AMF.

The effects of AMF on nutrient transformation processes in the soil are not well understood, especially for N. There is a urgent need to conduct process based studies, to fully understand how AMF affect nutrient cycling and how they could be managed to exploit their potential to promote sustainable nutrient cycles.

We conclude that AMF can contribute to efficient nutrient recycling. While the beneficial effects on P cycling appear to be relevant over a wide range of environmental conditions, their effects on N cycling seem to be much more context dependent.

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Supporting Information

Table S1: Mean values of the measured response variables of grassland microcosms filled with two different soil types (Pasture and heath soil) combined with two N fertilizers (NH_4^+-N and NO_3^--N) and either inoculated with AMF (M) or receiving a non-mycorrhizal control inoculum (NM). Numbers in brackets indicate ± 1 SE (n=7; for leaching of NH_4^+-N, NO_3^-- N and unreactive P, n is partially lower because of datapoints below the detection limit); ND: not detectable.

	Pasture soil				*Heath soil*			
	NH4-Fertilization		NO3-Fertilization		NH4-Fertilization		NO3-Fertilization	
leaching losses	M	NM	M	NM	M	NM	M	NM
NO_3^--N [mg]	0.79 (0.54)	2.4 (2.27)	13.5 (3.83)	12.5 (2.97)	ND	ND	32.7 (2.80)	29.7 (3.17)
NH_4^+-N [mg]	0.14	0.5 (0.07)	0.2 (0.02)	0.9 (0.16)	0.3 (0.08)	0.7 (0.44)	0.4 (0.10)	1.3 (0.46)
DON [mg]	10.40 (1.05)	7.5 (0.78)	5.9 (1.10)	6.7 (0.87)	15.5 (1.57)	19.6 (0.70)	15.4 (2.72)	21.0 (2.03)
Total N [mg]	11.22 (1.08)	10.3 (2.46)	19.5 (3.53)	20.1 (3.31)	15.8 (1.54)	20.2 (0.92)	48.4 (4.30)	52.0 (5.23)
reactive P [mg]	0.06 (0.02)	0.1 (0.01)	0.1 (0.01)	0.1 (0.01)	ND	0.2 (0.08)	ND	0.2 (0.04)
unreactive P [mg]	0.09 (0.01)	0.1 (0.02)	0.1 (0.01)	0.1 (0.02)	0.2 (0.03)	0.3 (0.04)	0.2 (0.03)	0.3 (0.02)
Total P [mg]	0.14 (0.03)	0.2 (0.02)	0.1 (0.01)	0.2 (0.03)	0.2 (0.03)	0.3 (0.08)	0.2 (0.03)	0.4 (0.04)
N_2O- fluxes								
N_2O-flux [ng*m^{-2}*s^{-1}]	9.06 (2.66)	20.9 (7.11)	94.1 (39.09)	273.6(61.40)	1.9 (1.09)	2.0 (0.93)	199.8(35.25)	282.9 (33.58)
plant parameters								
Total biomass [g]	46.10 (3.70)	36.7 (2.26)	43.8 (4.03)	36.9 (1.39)	34.4 (2.00)	38.7 (2.92)	36.4 (4.48)	37.8 (2.49)
Plant N [mg]	1088 (43.10)	908 (42.92)	1003 (24.67)	951 (51.85)	498.0(16.29)	544.3(41.22)	514.1(39.91)	566.9 (32.72)
Plant P [mg]	101.0 (7.01)	79.8 (6.02)	91.4 (5.11)	78.8 (5.15)	84.7 (5.45)	73.1 (5.55)	77.7 (4.83)	77.6 (3.18)
Plant N:P ratio	11.00 (0.72)	11.6 (0.71)	11.1 (0.50)	12.2 (0.69)	6.0 (0.35)	7.5 (0.47)	6.6 (0.30)	7.3 (0.34)
soil parameters								
Mineral NO_3^--N [mg]	10.10 (3.52)	9.7 (4.05)	10.6 (3.48)	8.0 (2.49)	ND	ND	8.3 (1.16)	8.4 (0.72)
Mineral NH_4^+-N [mg]	5.67 (2.03)	5.1 (1.99)	3.9 (1.86)	5.3 (1.88)	28.8 (5.08)	19.6 (1.10)	5.2 (1.54)	6.4 (1.40)
Total soil N [g]	11.54 (0.66)	13.9 (2.40)	10.9 (0.92)	9.7 (0.75)	10.2 (0.26)	9.4 (0.27)	10.2 (0.33)	11.1 (0.47)
Available soil P [mg]	6.93 (0.69)	6.2 (0.49)	5.6 (0.41)	6.5 (0.33)	5.5 (0.44)	4.8 (0.37)	5.6 (0.52)	6.1 (0.76)
microbial biomass								
Microbial C [mg]	1835 (45.79)	1436 (137.9)	1677 (76.01)	1475 (113.8)	1455 (64.96)	1237 (113.0)	1646 (75.60)	1433 (79.58)
Microbial N [mg]	298.0 (9.00)	220.4(19.39)	272.1(15.06)	239.5(20.14)	165.2 (8.18)	146.7(13.98)	190.3 (8.34)	167.3 (8.00)
AMF root colonization								
HC (%)	50.43 (7.00)	0 (0.00)	64.1 (5.07)	0 (0.00)	12 (2.36)	0 (0.00)	12.7 (1.61)	0 (0.00)
AC (%)	5.57 (1.90)	0 (0.00)	8.29 (1.19)	0 (0.00)	0.57 (0.20)	0 (0.00)	0.14 (0.14)	0 (0.00)
VC (%)	14 (1.43)	0 (0.00)	20 (2.88)	0 (0.00)	6 (1.57)	0 (0.00)	6 (1.18)	0 (0.00)

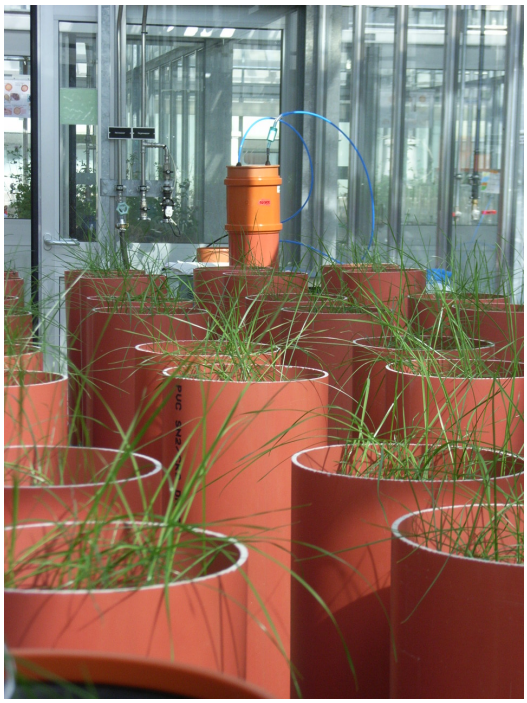

Figure S1: Grassland microcosms planted with Lolium multiflorum. In the back, the headspace of a microcosm is closed to measure N_2O fluxes.

Chapter 3

The role of the arbuscular mycorrhizal symbiosis in sustainable N cycling and denitrification

S. Franz Bender, Faline Plantenga, Franz Conen, Mark Rollog and Marcel G.A. Van der Heijden

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Abstract

Nitrogen is constantly accumulating in the biosphere due to human activities, representing a major threat to the earth ecosystem. To reduce the negative impacts of human perturbation on the N cycle, it has been proposed to focus on increasing N fertilizer use efficiency, reducing the transport of reactive N to rivers and groundwater and to maximize denitrification to its unreactive N_2 endproduct. However, little is known about the ecological interactions affecting these process.

The arbuscular mycorrhizal (AM) symbiosis is formed between a widely distributed group of soil fungi and the majority of land plants and has been reported to provide a number of beneficial ecosystem services including improved plant nutrition and reduced nutrient leaching losses from soil, as well as reduced N_2O emissions from denitrification. However, up to now, little is known about the effects of AMF on improving N-use efficiency and it remains unknown if AM fungi can also affect the emissions of N_2 and, hence, the removal of reactive N from the biosphere.

We set up a greenhouse experiment using tomato-mutants to manipulate the presence of AM fungi. We investigated the influence of AM fungi on plant N nutrition, leaching losses and denitrification, including measurements of N_2.

AMF strongly enhanced the N use efficiency by increasing plant N uptake and reducing N leaching losses. Moreover, N_2O emissions were reduced. Moreover, our results suggest that the AM symbiosis might promote complete denitrification to N_2.

This is the first attempt to asses a potential effect of the AM symbiosis on N_2 emissions. The results imply that the AM symbiosis might be able to promote emissions of unreactive N_2 from soil, hence removing excess reactive N with known detrimental effects on the earth ecosystem from the

biosphere. As AMF also improved plant nutrition, reduced nutrient leaching loss and N_2O emissions, we conclude that AMF play an important role for sustainable N cycling.

Introduction

Nitrogen is an essential element for life. Most of the global N occurs in the form of dinitrogen gas (N_2) which makes up nearly 80% of the earth's atmosphere. However, in this form, N is unreactive and is not directly available to plants or animals. Only by the activity of some microorganisms or through high-temperature processes, like flashing, it can naturally be transformed into reactive, hence bioavailable forms (Galloway et al., 2003). Since the development of the Haber-Bosch process, that allows the industrial production of mineral N fertilizers, the input of reactive N into the biosphere has nearly doubled (Schlesinger, 2009). This allowed great increases in crop production but also lead to high costs on the environment. Hence, the perturbation of the N cycle is considered one of the major threats to global sustainability (Rockstrom et al., 2009).

It has been estimated, that in agricultural systems about 55% of the N input is taken up by crops, while the remaining 45% are lost through leaching, soil erosion or gas emissions (Liu et al., 2010). These nutrient losses result in severe environmental threats like pollution of waterways and increases in atmospheric greenhouse-gas concentrations, N-depositionand biodiversity loss (Bobbink et al., 2010; Galloway et al., 2003).

It has been proposed that policy makers and scientists should focus on increasing fertilizer-N-use-efficiency, reducing N transport to waterways and maximizing denitrification to its N_2 endproduct (Schlesinger, 2009). Denitrification is a microbial respiratory process that reduces nitrogen oxides (NO_3^-, NO_2) to the gaseous products N_2O, a strong greenhouse gas, and N_2 (Philippot et al., 2007). Complete denitrification to N_2 is the only process, that can remove reactive N from the biosphere and transform it back to its non-reactive form (Seitzinger et al., 2006). Complete denitrification of nitrogen oxides in the soil to N_2, rather than incomplete denitrification to N_2O depends on number of abiotic factors interacting with each other, like N availability, C availability, O_2 availability/soil water

content, soil pH, soil C-to-N ratio and soil texture (Cuhel et al., 2010; Firestone et al., 1980; Gaskell et al., 1981; Senbayram et al., 2012). For instance, a high availability of organic C can reduce the N_2O/N_2 ratio when NO_3^- availability in soil is low, but can strongly enhance the N_2O/N_2 ratio when NO_3^- availabilbity in soil is high (Weier et al., 1993).

Soil microorganisms are major players in nitrogen (N) cycling, as they conduct almost the entire variety of N transformation processes (Robertson and Groffman, 2007). Arbuscular mycorrhizal fungi (AMF) are a very widespread group of microorganisms. They are obligate plant symbionts and are known to improve plan nutrition. Recently, their role in the N cycle received increasing attention (Hodge and Fitter, 2010; Smith and Smith, 2011; Veresoglou et al., 2012a). It has been reported that those fungi can take up inorganic N from soil and transport it to plants or immobilize it in their own biomass (Cavagnaro et al., 2012; Frey and Schuepp, 1993; Govindarajulu et al., 2005; Hodge and Fitter, 2010; Johansen et al., 1993; Mäder et al., 2000).

In greenhouse experiments, it has been shown that AMF can reduce the leaching of nutrients, including N (Asghari and Cavagnaro, 2012; van der Heijden, 2010). A large scale correlative study by de Vries et al. (2013) suggests that that reduced N leaching with increased AMF abundance is also relevant in the field.

Moreover, recent work indicates, that AMF also can play a role in reducing denitrification-related N_2O emissions from soil (Bender et al., 2013)

It remains, however, unknown whether N_2 emissions are also affected by AMF. The study of Bender et al. (2013) found a positive correlation of AMF abundance with the gene copy numbers of the functional gene nitrous-oxide-reductase (*nosZ),* which reduces the greenhouse gas N_2O to non-reactive N_2. It has been shown that a reduction in organisms containing the nosZ gene can affect the denitrification product ratio of N_2O/N_2, by increasing N_2O emissions while reducing N_2 (Philippot et al., 2011). This could imply, that an AMF-induced reduction in N_2O emissions is accompanied by an enhanced completion of the denitrification process, resulting in enhanced N_2 emissions. As denitrification to N_2 is the only process that can counteract the harmful accumulation of reactive N

species in the biosphere (Galloway et al., 2003), this would be a highly desirable, hitherto unknown trait of the arbuscular mycorrhizal symbiosis.

We set up an greenhouse experiment to test, how AMF affect the distribution of N in the plant soil system, including leaching losses with draining soil waterand gaseous losses as N_2O and N_2 to the atmosphere. We hypothesized that AMF (i) increase N uptake from soil and improve plant N nutrition, (ii) reduce N leaching into the groundwater and (iii) affect denitrification by reducing emissions of the greenhouse gas N2O and enhancing the emissions of the non-reactive N_2, resulting in enhanced denitrification efficiency.

Material and Methods

The experiment was conducted in microcosms constructed from PVC tubes with a diameter of 15 cm, a height of 40 cm, and a volume of approx. 7 L. A drain tap was inserted in the bottom of the tubes to allow leachate collection. A sleeve with a rubber seal and a removable cap was fit on the tubes to close the headspace airtight. For N_2O and CO_2 analyses, the cap contained two valves in which tubes for gas sampling could be inserted. For N_2 sampling, another cap was used, which contained a valve with a rubber septum through which headspace gas samples could be taken with a syringe. The sleeve could be moved vertically along the tube surface to form the headspace chamber. For better drainage and filtering purposes, 1250 g of an autoclaved sand-gravel mixture was added to the bottom of the tubes.

The soil was collected from a regularly manured long-term pasture on an organic farm near the Research Station Agroscope ART in Zürich, Switzerland (47°43`11.83`` N, 8°53`65.25`` E). The soil had a sandy-loam texture, was moderately-drained and characterized as *calcaric cambisol.* The soil is regularly subjected to short periods of waterlogging under wet weather conditions. It was sieved through a 5 mm sieve to homogenize and to remove large stones, plant material, earthworms and other macrofauna that could cause undesired variation. Microcosms were filled with 6L of the sieved field soil. In addition to this, 550 ml of an additional AMF inoculum was mixed with this soil to assure

a high AMF root infection potential. The characteristics of the soil-inoculum mix are shown in Table 1. The inoculum was a complex inoculum produced from the same field soil used to fill the microcosms and served to amplify the indigenous AMF community. It has been produced in 3 L pots, containing a 3:17 (v/v) soil:sand-mixture planted with *Plantago lanceolata*. The soil:sand-mixture had been inoculated with 5 % fresh field soil. Every 2nd week, pots received 20 ml of a modified Hoagland solution (Hoagland and Arnon, 1950), containing one quarter of the original P concentration. After three months of growth, pots were dried, emptied, and roots were cut into <5 cm pieces and homogeneously mixed with the substrate.

Table 1: Characteristics of soil-inoculum mixture used to fill the microcosms.

Substrate characteristics	
Clay [%]	22.6
Silt [%]	30.9
Sand [%]	43.4
Humus [%]	3.1
pH (H_2O)	7.3
C_{org} [g*kg soil^{-1}]	18.0
N total [g*kg soil^{-1}]	28.0
available P [mg*kg soil^{-1}]	0.99

Two genotypes of Tomato (*Solanum lycopersicum L.* cv. Micro-Tom), the BC1-mutant (NM treatment) and its progenitor wild-type, were planted into the microcosms. The BC1-mutant exhibits a strongly reduced AMF root colonization compared to its` wildtype progenitor (M treatment) (Meissner et al., 1997). This mutant/wildtype pair was created by fast-neutron mutagenization (David-Schwartz et al., 2001) and hybridization and has been demonstrated to be very suitable for studies in AMF ecology (Rillig et al., 2008). The tomato seeds were germinated in a sterilized 1:1 (v/v) sand-soil mixture and then transplanted into the microcosms. A test for equal performance of both tomato genotypes in

absence of AMF was conducted and is described in (Bender et al., 2013). This test did not reveal any significant differences among the genotypes in absence of AMF.

The plants were grown in a greenhouse with an average daily temperature of 24 ºC, nightly temperature of 18 ºC and 16 hours of light per day. Supplemental light was provided by 400 W high-pressure sodium lights when natural irradiation was lower than 300W/m^2. Plants were regularly watered to 40% WFPS with deionized water. The experiment consisted of two treatments, the M treatment planted with the wildtype, and the NM treatment planted with the BC1-mutant, each replicated 10 times and was established in three randomized blocks. The blocks were set up in two week intervals, starting July 26, 2011. The microcosms are pictured in Fig. 1.

When filling the microcosms, substrate dry weights were determined gravimetrically. The exact weight of the pots was noted to be able to calculate the water filled pores space (WFPS) as described below.

Figure 1: Microcosms filled with soil and planted with 2 different Tomato genotypes. For the central microcosm, the headspace is closed for the measurement of N2-emissions (see Material and methods for details).

Fertilization and Water Pulse

After 10 weeks of plant growth, the microcosms were watered to 94% WFPS with deionized water mixed with 10ml of nutrient solution containing 778 mM ^{15}N-labeled KNO_3 (60 atom% excess), 59 mM KH_2PO_4, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.5 µM $CuSO_4$ and 0.5 µM Na_2MoO_4. This corresponded to a fertilizer pulse of 60 kg N ha^{-1} and 10 kg P ha^{-1}. The higher water and nutrient loadings were introduced to provide conditions conducive for denitrification. After fertilization, gas fluxes were measured.

Gas sampling

To measure the fluxes of N_2O and CO_2 from the microcosms, the headspace was adjusted to a height of 20cm above soil surface (4 L volume) and closed for a period of 10 minutes with the headspace gas pumped through a sample loop, first into a LI-820 CO_2 Gas Analyzer (LI-COR Biosciences, Lincoln, US) and, subsequently, to a TEI46c automated N_2O analyzer (Thermo Fisher Scientific, Waltham, US). After N_2O and CO_2 measurements, the headspace was opened, aerated for 30 min and N_2 emissions were measured. For the N_2 analyses, the headspace was reduced to a height of 11 cm and a volume of approximately 2 L. For taking samples for N_2 analysis, the headspace was kept closed for 3 hours (Fig.1) to allow the accumulation of $^{15}N_2$ concentrations high enough to reliably be distinguished from the atmospheric N_2 background. Before sampling, headspace air was ventilated for 5 min with a fan installed on the inside of the removable cap. After 3 hours a 1 ml headspace gas sample was taken with an airtight syringe and transferred to a 12 ml Exetainer (Labco, High Wycombe, GB) for later analyses. The cap used to close the headspace was non-transparent.

At every gas sampling, the respective pot was weighed to determine the actual WFPS.

Gas fluxes were measured every 12 hours for a period of 7 days resulting in 14 flux measurements per microcosm.

Rainfall simulation and Harvest

After the gas measurement period, the pots were watered to 80 % water holding capacity and, subsequently, received an artificial rainfall of 1l with a rain simulator as described by Knacker *et al.*

(2003). The drain tap in the bottom of the microcosms was opened so that the leachate could be collected. After approximately 2 h, when leaching had ceased, the leachate was weighed and 2 50ml subsamples were taken. One subsample was cooled at 4°C and the other one was frozen until analysis.

The shoots where cut at soil surface, dried at 60° C, and weighed. The microcosms were emptied and the roots where collected, rinsed with water, cut into pieces < 2 cm, and a subsample of 1-2 g was taken and stored in 50 % ethanol. Remaining roots were dried and weighed. The remaining substrate was mixed thoroughly, and soil samples were taken. Soil water content at harvest was determined gravimetrically to relate all soil analyses to soil dry matter

Laboratory Analyses

N_2

N_2 samples were analyzed with isotope ratio mass spectrometry (IRMS) according to the ^{15}N gas-flux method as described in Stevens and Laughlin (1998). Samples were flushed out of the Exetainer with helium and transported to a modified Finnigan Gasbench II (Finnigan, Bremen, DE), where compounds that could affect the analyses were removed. Total N_2 content and isotopic composition of the N_2 molecules were analyzed with a Thermo Finnigan Delta plus XP Mass spectrometer (Finnigan, Bremen, DE).

The mass spectrometer output was manually corrected for machine drifts and the ratios ^{29}R ($^{29}N_2/^{28}N_2$) and ^{30}R ($^{30}N_2/^{28}N_2$) of the N_2 in the samples were calculated. The measurements of $^{30}N_2$ yielded uninterpretable data, as, NO had been formed in the ion-source of the mass-spectrometer, either from N_2 and O_2, or from N_2 and oxidation in the filament and source components. As NO also has a molar mass of 30, it interferes with the $30N_2$ measurements. Thus, N_2 emissions could not adequately be quantified. However, data for $^{29}N_2$ were still interpretable and could be quantified as

$$^{29}N_{2=}(^{29}R_{sample} - {}^{29}R_{atmosphere})*N_{2headspace}$$

where $^{29}N_2$ is the mass $^{29}N_2$ emitted during the measurement, $^{29}R_{sample}$ is the ratio of $^{29}N_2/^{28}N_2$ in the enriched sample, $^{29}R_{atmosphere}$ is the ratio of $^{29}N_2/^{28}N_2$ in the atmosphere (0.0036782) and $N_{2headspace}$ is the amount of N_2 present in the headspace air.

If we assume that the ratio $^{29}N_2$ and $^{30}N_2$ is the same among the treatments, we can make a relative comparison between N_2 emissions of both treatments but no quantification of total N_2 loss.

Leachate

Leachates were chemically analyzed for nutrient concentrations. NO_3^--N, NO_2-N and dissolved PO_4^{3-}-P were determined using a Dionex DX500 anion chromatograph (Dionex Corporation, Sunnyvale, CA). Total P in leachate was determined using Oxisolv (Merck, Darmstadt, DE) oxidation prior to the photometric analysis using the molybdenum blue ascorbic acid method (Watanabe and Olsen, 1965). Ammonium was analyzed photometrically after reaction with salicylate and dichloroisocyanuric acid using a Skalar segmented flow analyser (Skalar, Breda, NL). The measured nutrient concentrations were multiplied with the leachate volume to get the total nutrient loss per microcosm. Amounts of NO_2-N were low and were added to NO_3^--N values.

Plant and soil N contents

Dried shoots and roots were ground with a centrifuge mill (0.12 mm), a dried soil subsample was milled in a ball mill and their total N content was determined with a FLASH Elemental Analyzer 1112 (Thermo Finnigan, Waltham, MA, USA).

Mineral soil N

Mineral soil N was analyzed after extraction with 0.0125M $CaCl_2$. Mineral soil NH_4^+ and NO_3^- concentrations in the extracts were then analyzed as described above for leachates.

Microbial Biomass C and N

Microbial Biomass C and microbial biomass N estimates by chloroform-fumigation-extraction (CFE) were carried out according to (Vance et al., 1987). CFE was done in duplicate on 20 g (dry matter) subsamples that were extracted with 80 ml of a 0.5 M K_2SO_4 solution. Organic C was determined by infrared spectrometry after combustion at 850°C (DIMATOC® 2000, Dimatec, Essen, DE). Total N was

subsequently measured in the same sample by chemoluminescence (TN_b, Dimatec, Essen, DE). Soil microbial biomass C and N was then calculated according to (Jörgensen and Mueller, 1996).

AMF-colonized root length

The percentage of root length colonized by AM fungi was analyzed after clearing the roots with 10% KOH and staining them with a 5 % pen ink in vinegar mixture (Vierheilig *et al.* 1998), using a modified line-intersection method (McGonigle *et al*., 1990). 100 Intercepts were counted per sample.

AMF extraradical hyphal length

The length of extraradical fungal hyphae in the soil was determined by a modified aqueous extraction and membrane-filter technique (Jakobsen et al., 1992) on duplicate samples of 2 g. Hyphal length was calculated according to the modified Newman formula for calculating root length (Tennant, 1975).

Water filled pore space (WFPS)

Particle density of the soil was analyzed with a water-pycnometer according to the reference methods of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL et al., 1996). The particle density was determined to be able to calculate the WFPS in the microcosms as described in Elliott et al. (1999) but using the actual particle density determined from our substrates., WFPS declined nearly linearly in the microcosms during the gas measurement period, and the slope of the linear decline was used to statistically test for treatment effects.

Statistical analyses

All data were analysed with two-way ANOVA, including the block effect and the Tomato genotype as factors. Model residuals were checked for normality and homoscedasticity by plotting fitted values against residuals and data was log-transformed where necessary. Non-parametric Kruskal-wallis tests were performed to analyse treatment differences of AMF root colonization measures.

All analyses were done with the R-statistical software, version 3.0.1.

Results

AMF performance

Root colonization of the wild type tomato (M treatment) was on average 43% and it was two-fold higher then root colonization of tomato mutants of the NM treatment, which was 19%. (Table 2).

Plant biomass and N contents

Shoot biomass was significantly enhanced in the M treatment compared to the NM treatment, while root biomass was low and did not differ between the treatments (Table 2). The total shoot N content was significantly enhanced by 73% in the WT-tomatoes of the M treatment compared to the mutants of the NM treatment. Root N contents were much lower and did not differ between the treatments (Fig. 2, Table 2).

Soil N contents

Soil mineral NO_3^- content at the end of the experiment was significantly reduced by 11% in the M treatment compared to the NM treatment. No differences in the mineral NH4 content and total soil N between the treatments were detected (Table 2).

Microbial biomass C and N contents

No significant difference in the microbial biomass C and N contents or the Microbial biomass C/N ratio could be detected between the treatments (Table1).

WFPS

WFPS did not differ significantly between the microcosms and declined at similar rates during the gas measurements (Table 2).

Table 2: Mean values of plant, soil, leaching and gas emissions and F-statistics for the effect of tomato-genotype in the two-way ANOVA. Numbers in brackets indicate ±1SEM.

	WT		MT		*F-value*	*P-value*
Plant biomass						
shoots [g]	4.50	(0.249)	2.85	(0.294)	***24.13***	***<0.001***
roots [g]	0.57	(0.100)	0.48	(0.071)	*0.70*	*0.416*
total [g]	5.07	(0.265)	3.33	(0.289)	***37.35***	***<0.001***
Plant N content						
shoots [mg]	175.0	(9.03)	96.5	(9.51)	***44.8***	***<0.001***
roots [mg]	10.82	(1.980)	10.78	(1.631)	*0.00*	*0.987*
total [mg]	185.8	(9.247)	107.3	(9.245)	***58.84***	***<0.001***
Soil						
minNH_4-N [mg]	1.48	(0.146)	1.44	(0.149)	*0.10*	*0.754*
minNO_3^--N [mg]	77.30	(5.191)	86.46	(6.064)	***5.95***	***0.027***
total soil N [mg]	15139	(1017)	14032	(529.9)	*0.89*	*0.361*
water removal [%WFPS h^{-1}]	-0.050	(0.002)	-0.049	(0.003)	*0.05*	*0.829*
Microbial biomass						
MBN [mg]	642.7	(11.08)	635.2	(14.93)	*0.14*	*0.71*
MBC [mg]	4631	(66.76)	4653	(84.75)	*0.06*	*0.803*
MBC/N ratio	7.22	(0.136)	7.35	(0.188)	*0.3777*	*0.548*
Nutrient leaching						
NH_4^+-N [mg]	0.31	(0.049)	0.33	(0.050)	*0.20*	*0.663*
NO_3^--N [mg]	105.3	(14.37)	158.6	(16.81)	***27.93***	***<0.001***
Gas emissions						
N_2O-N [mg]	3.06	(0.386)	4.14	(0.246)	***6.52***	***0.021***
$^{29}N_2$ [mg]	2.51	(0.291)	1.85	(0.226)	***7.31***	***0.016***
product ratio [N_2O-N/$^{29}N_2$]	1.38	(0.225)	2.55	(0.322)	***24.06***	***<0.001***
CO_2 [mg]	631.9	(40.48)	496.6	(37.35)	***6.30***	***0.033***
AMF parameters						
extraradical hyphal length [m g $soil^{-1}$]	6.30	-(0.76)	5.26	-(0.52)	*1.54*	*0.232*
root length colonized*					χ^2	*P-value*
hyphae [%]	43.40	(3.40)	18.90	(1.93)	***14.31***	***<0.001***
vesicles [%]	1.70	(0.50)	0.00	(0.00)	***9.67***	***0.002***
arbuscles [%]	38.10	(3.01)	9.90	(1.45)	***14.32***	***<0.001***

*a non-parametric Kruskal-wallis-test was used

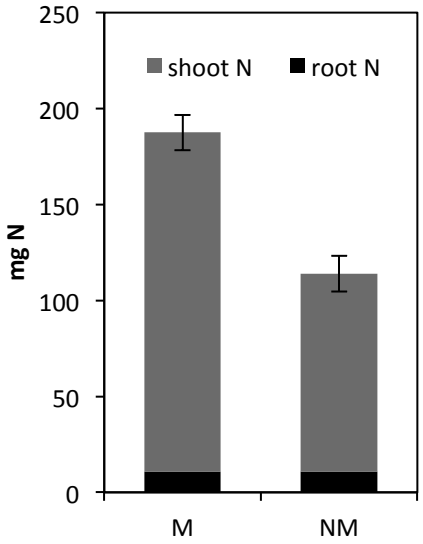

Figure 2: N contents in shoot and root biomass of the mycorrhizal tomato-wildtype (M) and the non-mycorrhizal tomato-mutant (NM). Error bars indicate ±1SEM.

Leaching losses

Leaching of NO_3^--N was significantly reduced by 34% in the M treatment compared to the NM treatment (Fig. 3). Leaching of NH4 was much lower and did not differ between treatments.

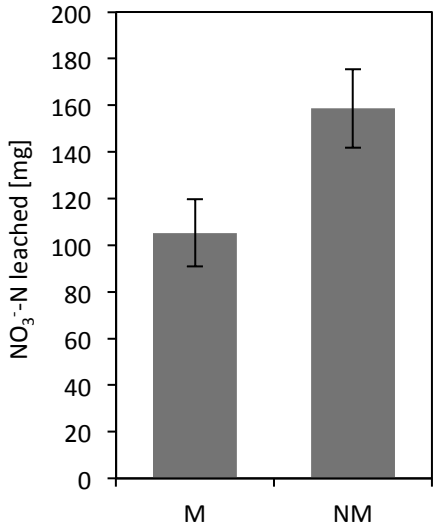

Figure 3: Amount of NO_3^- leached from soil planted with the mycorrhizal tomato-wildtype (M) and the non-mycorrhizal tomato-mutant (NM). Error bars indicate ±1SEM.

Gas emissions

Until 36h after fertilization, the fluxes of N_2O did not differ between the treatments but, thereafter were lower in the M treatment until the end of the measurements (Fig. 4a). Cumulative N_2O emissions were significantly reduced by 26% in the M treatment compared to the NM treatment (Fig. 5). The fluxes of the quantifiable fraction of N_2 emitted were similar among both treatments until 60 h after fertilization. Thereafter, $^{29}N_2$ fluxes were higher in the M treatment compared to the NM treatment (Fig. 4b)

The cumulative emissions of $^{29}N_2$, were significantly increased by 36% in the M treatment (Fig. 5).

The resulting N_2O-N/$^{29}N_2$ denitrification product ratio was significantly reduced by 46% in the M treatment (Fig. 6).

Cumulative emissions of CO_2 were significantly increased by 27% in the M treatment (Table 2).

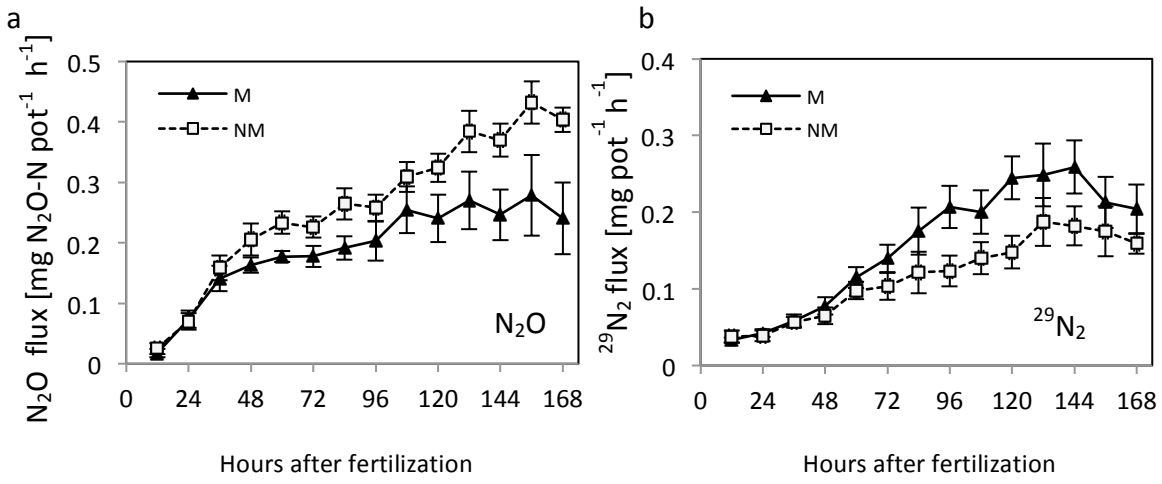

Figure 4: Fluxes of N_2O and $^{29}N_2$ from soil planted with the mycorrhizal tomato-wildtype (M) and the non-mycorrhizal tomato-mutant (NM). Error bars indicate ±1SEM.

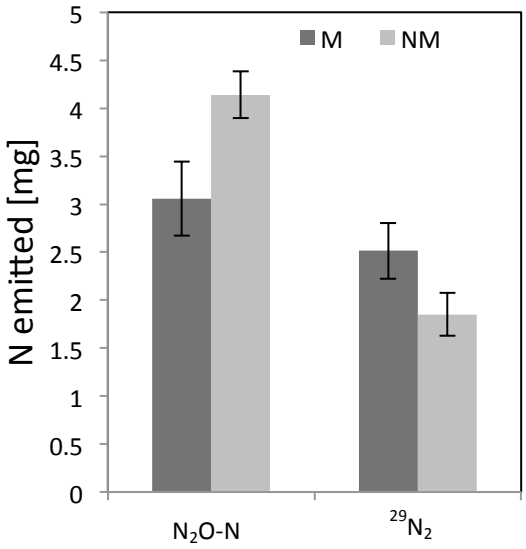

Figure 5: Cumulative N_2O-N and $^{29}N_2$ emissions from soil planted with the mycorrhizal tomato-wildtype (M) and the non-mycorrhizal tomato-mutant (NM). Error bars indicate ±1SEM.

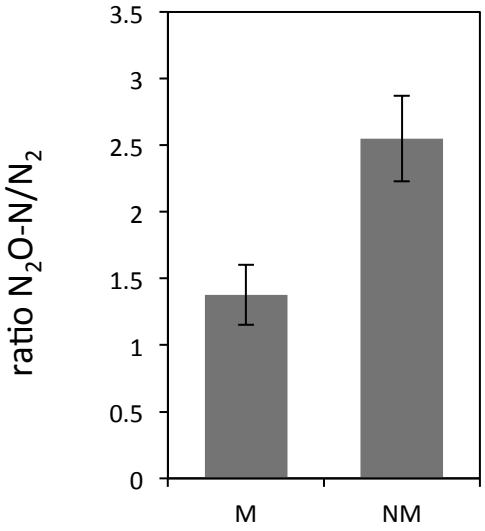

Figure 6: Denitrification N_2O-N / $^{29}N_2$ product ratio from soil planted with the mycorrhizal tomato-wildtype (M) and the non-mycorrhizal tomato-mutant (NM). Error bars indicate ±1SEM.

Discussion

The results presented here clearly show, that AM fungi can enhance the nutrient use-efficiency of N. Plant N contents were significantly increased, while soil mineral N pools and mineral N leaching losses simultaneously were decreased in the presence of AM fungi. As has been shown before, AMF contributed to reduced N_2O emissions. However, our data also suggest that AM fungi might increase N_2 emissions and hence the denitrification product ratio of N_2O/N_2. To our knowledge, this is the first report addressing the impact of AM fungi on N_2 emissions.

The results indicate that besides AMF possessing the ability to induce a reduction of emissions of the strong greenhouse gas N_2O, they might also have the potential to promote complete denitrification to N_2 and therefore to promote the removal of excess reactive N from the biosphere, potentially hazardous for the environment.

When N2 is formed from a NO_3^- pool containing a certain ratio of 15N, the total N_2 emissions are composed from N_2 molecules with the masses 28 (N_2 molecule composed of two 14N atoms), 29 (N_2 molecule composed of one 14N and one 15N atom) and 30(N_2 molecule composed of two 15N atoms). Total N_2 emitted can then be quantified by measuring all three N_2 species in the headspace air and applying certain equations and approximations (Mulvaney and Boast, 1986).

However, due to technical problems, we could not quantify the fraction of $^{30}N_2$, and, hence, we cannot quantify total N_2 emissions. If we assume that the NO_3^--pool in the soil (composed of native soil NO_3^- and 60% 15N labeled fertilizer NO_3^- added) being denitrified had a similar ratio of 15N/14N atoms (R_{soil}) in both treatments, we can make a relative comparison of N_2 emissions between treatments based on our $^{29}N_2$ data. It is, however, possible that that less mineral soil N was present in the M treatments already at the time of fertilization, because of higher plant N uptake. If this was the case, then the addition of the 15N labeled fertilizer might have resulted in a higher R_{soil} in the M compared to the NM treatments. A higher R soil could also increase the fraction 29N2 molecules from total N2 emissions even if total N2 emissions were equal among the treatments. Consequently, the results on N2 emissions presented here have to be interpreted cautiously.

A wide range of mechanisms can be responsible for the observed findings. It has been shown before, that AMF rooting systems can enhance N interception from soil, improve plant N nutrition and reduce N leaching losses (Asghari and Cavagnaro, 2011; Cavagnaro et al., 2012; Johansen et al., 1993; Mäder et al., 2000). In our study, root biomass did not differ between treatments, indicating that AMF extraradical hyphae probably enhanced N interception from soil leading to the strongly increased shoot N contents in the M treatment. Increased N interception by AMF is also likely to be the main cause for the reduced soil mineral N contents and reduced N leaching losses found in the M treatment compared to the NM treatment at the end of the experiment.

It has been shown that increasing soil NO_3^- concentrations increase the N_2O/N_2 ratio and that NO_3^- can directly inhibit the reduction of N_2O to N_2 (Firestone et al., 1980; Gaskell et al., 1981; Senbayram et al., 2012). Consequently, the higher availability of mineral NO_3^- in the soil, due to reduced N uptake in the NM treatment could have directly affected the N_2O/N_2 ratio positively. Besides NO_3^-, also increased organic C availability has been shown to promote N_2O emissions relative to N_2. AMF were shown to affect the C allocation of plants into soil and to provide a strong carbon drain away from the rhizosphere (Drigo et al., 2010; Jakobsen and Rosendahl, 1990), where denitrification activity is assumed to be highest (Giles et al., 2012; Mahmood et al., 1997). Reduced plant rhizodeposition in the presence of AMF has also been reported (Graham et al., 1981). Hence, the presence or increased abundance of AMF in the M treatment might have reduced the availability of organic C to the denitrifying soil community, resulting in a reduced N_2O/N_2 product ratio. In accordance with the literature, CO_2 emissions were also increased in the M treatment, indicating that AMF acted as carbon drain in the present study (Johnson et al., 2002). It has been reported, that denitrifying communities located in non-rhizosphere bulk soil predominantly produce N_2 as denitrification endproduct, compared to denitrifying communities located in the rhizosphere soil that predominantly produce N_2O (Cheneby et al., 2004). A reduction of C input into the rhizosphere (Graham et al., 1981)and enhanced C transport into bulk soil through C exudation by AMF hyphae (Hooker et al., 2007; Toljander et al., 2007) could therefore promote N_2 production while reducing

N_2O emissions. In the study of Bender et al. (2013), a reduction in the abundance N_2O producing nirK-gene had been observed with increasing AMF abundance, while simultaneously a reduction in the abundance of N_2O reducing, hence N_2 producing nosZ gene had been found supporting this potential mechanism. However, more targeted experiments have to be performed to specifically test this.

These findings would match with our observations that N_2O emissions were reduced, while N_2 emissions were increased in the presence of AMF.

Another factor known to affect the denitrification product ratio is O_2 availability. Firestone et al. (1980) reported relatively higher N_2O emissions with increased O_2 availability.

No difference in WFPS and water removal from soil could be detected between the treatments, indicating no differences in O2 availability due to soil water content.

However, the increased CO_2 emissions in the M treatment could be an indication towards higher, heterotrophic respiration in soil, hence reducing O_2 availability. However, CO_2 emissions also include plant respiration and do not account for photosynthetic CO_2 uptake, as the headspace chamber was constructed from non-transparent material and plants were in the dark during CO_2 measurements.

Consequently, no clear conclusions on differences in O_2 availability in soil between the treatments can be drawn but an effect on the observed changes in the N_2O/N_2 ratio cannot be excluded.

In a study comparing organically with conventionally managed apple orchards, Kramer et al. (2006) found that in organically managed apple orchards N leaching losses were reduced and denitrification efficiency (i.e. the complete reduction to N_2) increased. As apple trees associate with AMF and the abundance of AMF was shown to increase under organic management (Verbruggen et al., 2010), a higher abundance of AMF in the organically managed fields might have contributed to the effects observed in this study.

AMF affect ecosystem processes by various pathways, all being interrelated with each other (Rillig, 2004). Consequently, identifying the precise mechanisms by which the presence/abundance of AMF affects the N_2/N_2O denitrification product ratio is very challenging and rewards further research.

The completion of denitrification towards complete NO_3^- reduction to N_2 is the only process on earth removing reactive N from the biosphere which is constantly accumulating and representing a major threat to the earth system (Galloway et al., 2003; Rockstrom et al., 2009). To keep N cycling within acceptable boundaries, it has been proposed to apply measures to increase fertilizer use efficiency, reduce the transport of reactive N to rivers and groundwater and maximize denitrification to its N_2 endproduct (Schlesinger, 2009).

Here we show that AMF increase fertilizer use efficiency and present data that suggests that AMF might also maximize denitrification to its N_2 endproduct. Although, our data does, due to technical issues, not allow definite conclusions, our findings strongly warrant further research.

If a reduction of the N_2O/N_2 product ratio by AMF can be experimentally confirmed, this would open new perspectives for the management of global N cycling and global sustainability.

Chapter 4

Soil biota enhance agricultural sustainability by improving crop yield, nutrient uptake and reducing nitrogen leaching losses

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Abstract

1. Efficient resource use is a key factor for sustainable production and a necessity for meeting future global food demands. However, the factors that control resource use efficiency in agro-ecosystems are only partly understood.
2. We investigated the influence of soil biota on nutrient leaching, nutrient use efficiency and plant performance in outdoor, open-top lysimeters comprising a volume of 230 L. The lysimeters were filled with sterilized soil in two horizons and inoculated with a reduced soil-life inoculum (soil biota ≤11 µm, microbially dominated) and an enriched soil-life inoculum (soil organisms ≤2 mm, also containing arbuscular mycorrhizal fungi (AMF). A crop rotation was planted and nutrient leaching losses, plant biomass and nutrient contents were assessed over a period of almost two years.
3. In the first year of the experiment, enriched soil-life increased crop yield (+22 %), N uptake (+29 %) and P uptake (+110 %) of maize, and strongly reduced leaching losses of N (-51 %, corresponding to a reduction of 76 kg N* ha^{-1}). In the second year, wheat biomass (+17 %) and P contents (+80 %) were significantly increased by enriched soil-life but the differences were lower than in the first year.
4. Enriched soil-life also increased P mobilization from soil (+112 %) and significantly reduced relative P leaching losses (-25 %), defined as g P leached per kg P plant uptake, as well as relative N leaching losses (-36 %), defined as kg N leached per kg N plant uptake, demonstrating that nutrient use efficiency was increased in the enriched soil-life treatment.

5. *Synthesis and applications:* The results show that soil biota are a key factor determining resource efficiency in agriculture. The effects of management practices on soil biota must be seriously taken into consideration when aiming to enhance the sustainability of cropping systems.

Introduction

The high agricultural yields currently produced in many parts of the world are often achieved with the aid of excessive fertilizer use (Ju et al., 2009). Only about 50 % of N inputs to agricultural lands are used by crops, with a huge fraction of the inputs remaining unused for agricultural purposes and being lost through leaching and gas emissions (Liu et al., 2010; Smil, 1999). These nutrient losses are known to cause severe environmental problems like ground- and surface-water pollution and eutrophication, reduced biodiversity in ecosystems and to contribute to global warming (Galloway et al., 2003; Schlesinger, 2009). Moreover, these nutrients represent valuable resources, which might become limited in the near future. For instance, the globally available stocks of phosphate are expected to be depleted in the next 50-100 years (Cordell et al., 2009). There is an urgent need to change paradigms towards sustainable agricultural practices that aim to use applied resources as efficiently as possible to ensure sufficient yields and reduce environmental impacts (Schlesinger, 2009).

Most nutrient transformations in soil are performed by soil organisms. Through their activities they drive nutrient cycling and play an important role in determining whether nutrients are made available to plants, are stored in the soil, or are prone to being lost from the plant-soil system (Robertson and Groffman, 2007; van der Heijden et al., 2008). Several studies have addressed the importance of soil biota and their interactions for nutrient mineralization and plant nutrition (e.g. Ingham et al., 1985; Setälä and Huhta, 1991). Commonly, nutrient mineralization and plant nutrition is increased by faunal activities, but increased nutrient loss through leaching is also often reported (Bardgett and Chan, 1999; Setälä et al., 1990).

Particular attention is given to arbuscular mycorrhizal fungi (AMF), a group of soil fungi that live in symbiosis with the majority of land plants, including many agricultural crops. AMF can mobilize nutrients from soil, transfer them to their host plants and improve plant nutrition (Smith and Read, 2008). Exploration of a larger soil volume and efficient nutrient uptake are considered key mechanisms for the improvement of plant nutrition through AMF (Jakobsen et al., 1992). Many studies observed a positive effect of AMF on P nutrition. In contrast, the contribution of AMF to plant N nutrition is far less clear (Smith and Smith, 2011). It was shown that these fungi can reduce losses of P (Asghari et al., 2005; van der Heijden, 2010) and N (Asghari and Cavagnaro, 2012) through leaching. Enhanced nutrient interception of AMF rooting systems is considered a main mechanism for the reduction of nutrient leaching losses.

Interactions between soil fauna and AMF were reported to result in positive effects on plant biomass (Gange, 2000; Klironomos and Kendrick, 1995) and sometimes enhanced plant nutrition (Lussenhop, 1996). A synergistic effect of enhanced mineralization by soil fauna with enhanced nutrient interception by AMF rooting systems, as indicated by a study of Koller et al. (2013), could result in a highly efficient nutrient cycling machinery that enhances nutrient mobilization from soil resources and provides an effective uptake pathway of the mobilized nutrients to plants. If applied to agriculture, this effect would enhance agricultural sustainability by promoting internal nutrient cycling and reducing the need for external nutrient inputs. However, little is known about such interactive effects on nutrient cycling, plant nutrition, and especially nutrient losses under ecologically relevant conditions (e.g. field settings). The majority of investigations addressing these issues were conducted in small microcosms in the greenhouse with questionable ecological relevance and transferability to field situations (Kampichler et al., 2001; Read, 2002). The investigation of the effects of soil biota and their food webs on nutrient cycling in field settings is difficult, because removing soil biota to obtain adequate control treatments is rarely possible without strong perturbations of the whole soil ecosystem (Hunt et al., 1987).

Here, we assess the impact of soil biota on plant growth, nutrient use efficiency, and nutrient losses in outdoor-lysimeters. To enhance the scale of the experiment and improve ecological relevance, we used outdoor-lysimeters comprising a volume of 230 L. The lysimeters were filled with top- and sub-soil to imitate the natural soil profile. This approach provides a clear advantage to studies in much smaller microcosms, as side effects like root and hyphal growth constraints due to limited soil volume are reduced. We planted an agricultural crop rotation in lysimeters either inoculated with an enriched soil-life inoculum (soil organisms ≤2 mm, including AMF) or a reduced soil-life inoculum (soil biota ≤11 µm, microbially dominated). This enabled us to test whether soil foodweb complexity influences nutrient cycling (see methods for further information).

Plant nutrition, biomass and grain yield, and leaching losses of soil nutrients were monitored over a period of almost 2 years. We hypothesized that enriched soil-life (1) increases plant biomass, nutrient contents and crop yields, and (2) reduces the leaching losses of soil nutrients. Our study demonstrates that soil biota contribute substantially to agricultural sustainability by supporting plant nutrient uptake and plant yield and by reducing nitrogen leaching losses.

Material and methods

Lysimeter setup

The outdoor lysimeter facility used here was established in 1971 and consists of 32 lysimeters each with an inner diameter of 59 cm and a depth of 84 cm, resulting in a volume of approx. 230 L. The lysimeters consist of a polypropylene container inserted into a concrete body. A hole in the bottom of the container collects soil water drainage that is stored in 25 L plastic containers positioned in a closed cabinet under the lysimeter (Fig. 1 & Fig. S1 in Supporting Information). Before the start of the experiment, 16 lysimeters were emptied, cleaned, and sterilized by spraying with a 1.2 % active chlorine bleach solution and rinsing with water.

Six m^3 of a soil classified as *calcaric cambisol* was collected from a long-term pasture on an organic farm near the Agroscope Research Station in Zürich, Switzerland (47°43`11.83`` N, 8°53`65.25`` E).

The pasture has had manure regularly applied. Top- (0-30 cm) and sub-soil (30-80 cm) were collected separately and processed and sterilized as described in supporting methods (Appendix S1).

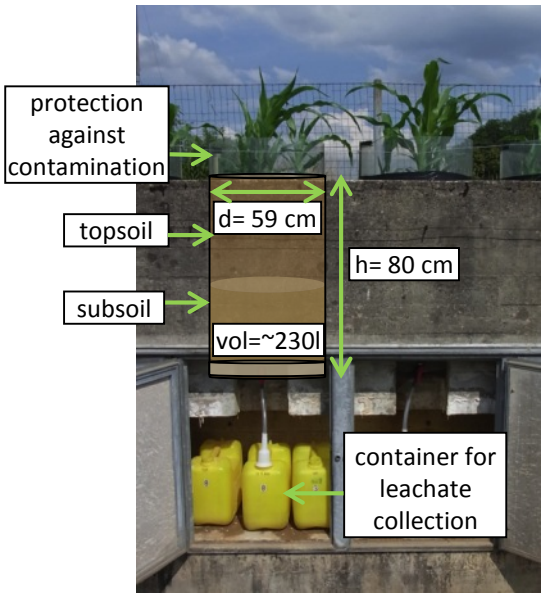

Figure 1:Setup of Lysimeters

Inoculum production

Two soil inocula were produced in the greenhouse by mixing a sterile sand: soil mixture with either fresh or autoclaved field soil (both 2mm-sieved). This was the same soil used to fill the lysimeters. Both inocula received a microbial wash (soil suspension, 11 µm filtered, see Appendix S1 for details). Pots were planted, grown in the greenhouse for 4 months, air-dried, and harvested (see Appendix S1 for details). The substrate in the pots inoculated with fresh field soil, including cut-up root pieces, was used to set up the enriched soil-life (ENR-) treatment containing soil organisms ≤2 mm (e.g. members of the soil meso- and microfauna, AMF, and microflora). The substrate with cut up root pieces produced from the autoclaved field soil was used to set up the reduced soil-life (RED-) treatment containing predominantly microorganisms ≤11 µm and some protozoa. The abundance of

various soil biota present in both inocula before lysimeter-filling was conducted by Earthfort Testing Services (Corvallis, OR, USA) and is shown in Table 1. 13 L of inoculum was added to each lysimeter. We focused on soil organisms ≤2mm as larger soil organisms, like earthworms, due to their lower number in soil, might have been distributed unequally among the different inoculum pots acting as a potential source of undesired variation. The size fraction of organisms ≤11 µm for the RED-treatment was chosen to provide a microbial community providing basic ecosystem functions but to exclude AMF. Moreover, a range of studies showed that larger soil biota (e.g. those bigger than 11 µm), including AMF, are more negatively affected by intensive agricultural management (e.g. intensive ploughing, reduced crop diversity and heavy fertilisation) compared to smaller sized organisms such as bacteria and fungi (Bradley et al., 2006; Postma-Blaauw et al., 2010; Wardle, 1995). The cultivation step in the greenhouse in nutrient poor substrate provided conditions favourable for the propagation of AMF in the ENR-treatment and served to establish a well-developed microbial community in both inocula. Despite the addition of a microbial wash, we cannot rule out the possibility that the microbial communities differed between the inoculums, as the presence or absence of other groups of soil organisms is likely to affect microbial communities.

Table 1: The abundance of bacteria, fungi and protozoa in the inoculum used to create the treatments as determined from a composite sample of all inoculum pots by Earthfort Testing Services (Corvallis, OR, USA). AMF root colonization was determined from 3 subsamples of a composite sample. Active bacteria and fungi were quantified by microscopy after staining with fluorescin diacetate. Total bacteria were quantified by microscopy using a fluorescein isothiocyanate method. Fungal biovolume was measured under the microscope and converted into total fungal biomass. Protozoa were quantified with a most probable number approach after direct counting. Nematodes were counted by microscopy. AMF root colonization was assessed as described in supporting methods (Appendix S1).

	enriched soil-life	reduced soil-life
microorganisms [µg g^{-1}]		
active bacteria	5.06	4.03
total bacteria	434	343
active fungi	0	0
total fungi	39.9	44.4
hyphal diameter [µm]	2.6	2.7
Protozoa [no g^{-1}]		
flagellates	8356	5785
amobae	13927	5785
ciliates	0	28
Nematodes [no g^{-1}]	0.02	0
AMF root colonization [%] (n=3)		
total	59.67 (6.17)	0 (0.00)
vesicles	13.33 (2.91)	0 (0.00)
arbuscles	15.33 (4.63)	0 (0.00)

Lysimeter filling

The lysimeters were filled as described in the supporting methods (Appendix S1). The characteristics of the subsoil and the topsoil-inoculum mixture filled into the lysimeters are presented in Table 2. The experiment was arranged in two blocks and comprised of 2 treatments each replicated 8 times.

Table 2: Characteristics of the sterilized sub-soil and top-soil used to fill the lysimeters

	top-soil	sub-soil
Clay [%]	25.05	26.50
Silt [%]	33.25	55.90
Sand [%]	38.70	17.30
Humus [%]	3.00	0.30
organic C [t ha^{-1}]	102.01	11.04
N total [t ha^{-1}]	12.79	2.30
available P [kg ha^{-1}]	5.331	0.358
P_{AAE} [kg ha^{-1}]	128.17	13.25
K_{AAE} [t ha^{-1}]	0.80	0.60
Mg_{AAE} [t ha^{-1}]	9.9	66.0
Ca_{AAE} [t ha^{-1}]	137.6	306.0
$CaCO_3$ [t ha^{-1}]	976.54	3069.12
pH (H_2O)	7.50	8.60

Planting

All seeds were surface sterilized before planting by stirring in 1.25 % bleach for 10 min. The crop rotation started with maize and was planted in May 2011. In July 2011, a grass-mixture was sown between the maize plants to provide AMF host plants over the winter. Maize was harvested in August 2011 and the grass-mixture in March 2012. Subsequently, summer wheat was sown and harvested four months later in July 2012. Finally, a grass-clover mixture was grown until January 2013. Details are given in supporting methods (Appendix S1).

Fertilization and watering

We expected considerable nutrient mobilization from the soil sterilization process and therefore did not fertilize in the first year. In the second year, wheat was fertilized with commercial NH_4NO_3 fertilizer corresponding to 50 kg N ha^{-1} applied on 16/04/2012 and 30 kg N ha^{-1} on 23/05/2012. Because of summer drought, the lysimeters were watered on 3 occasions between July and September in 2011 (a total of 25l per lysimeter) with tap water when maize plants were water stressed and turgor-loss was visible

Sampling

Sampling of leachates, roots for determining AMF colonisation, soil for microbial biomass C and N contents and of plant biomass was conducted as described in the supporting methods (Appendix S1).

Leachate analyses

Concentrations of NO_3-N, NO_2-N, PO_4-P, and SO_4 were determined by anion chromatography, and total P was determined photometrically after oxidation. NH_4 concentration was determined by continuous flow analysis, and total dissolved N (TDN) was measured by chemoluminescence (see supporting methods Appendix S1 for details). All nutrient concentrations were multiplied with the leachate volume to calculate the total amount of nutrients lost per lysimeter.

The difference between TDN and mineral N (NO_3-N, NO_2-N and NH_4-N) was considered as dissolved organic N (DON). The amount of PO_4-P in the leachates was labelled reactive P. The difference between total P and reactive P was labelled unreactive P. This fraction comprises all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates, and particulate inorganic material, e.g. clays (Daniel and DeLaune, 2009).

Leaching data is presented on a yearly basis with year 1 ranging from maize sowing in May 2011 until harvest of the grass-mixture in March 2012, and year 2 ranging from wheat sowing in March 2012 to grass-clover harvest in January 2013.

Plant nutrient concentrations

The dried plant material was weighed and P concentrations were analysed photometrically according to Watanabe and Olsen (1965) after dry ashing. N concentrations were analysed after combustion with an elemental analyser (varioMax CN, elementar, Hanau, DE).

Soil biological parameters

AMF root infection was quantified after staining using a modified grid-line intersection method on 100 intersections per sample (McGonigle et al., 1990). Soil microbial biomass C and N contents were analysed with chloroform fumigation extraction as described in the supporting methods (Appendix S1).

Soil analyses

Soil texture, organic C, humus, $CaCO_3$, soil pH, available soil P extracted with CO_2-saturated water, ammonium acetate-EDTA -extractable soil P (PAAE), K, Mg, K, Ca and Mg and total soil N were all analysed using standard methods according to the reference protocols of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL et al., 1996). Soil analyses at the end of the experiment were performed in the topsoil only.

For the whole experimental period, we calculated the amount of soil P that had been mobilized from initial non-AAE extractable soil P resources (P_{mob}) as

$$P_{mob} = (P_{plant} + P_{leached}) - (PAAE_{start} - PAAE_{end})$$

where P_{plant} is the amount of P in total plant biomass and $P_{leached}$ is the total amount of P leached during the whole experiment, $PAAE_{start}$ is the amount of PAAE at the start of the experiment and $PAAE_{end}$ is the amount of PAAE at the end of the experiment.

Statistical analyses

To test if the inoculation treatments had any overall effect on nutrient cycling and plant growth, a multivariate analysis of variance (MANOVA) was conducted for the complete experiment and for years 1 and 2 separately. The models included Block and Treatment as factors and total plant biomass, plant N- and P-contents, total N- and P-leaching and SO_4-leaching as dependent variables. Where the MANOVA rendered significant treatment effects, two-way ANOVAs, including the block effect and the inoculation treatment as factors were performed for all measured plant and nutrient

compounds to allow biological interpretation of the results. Model residuals were checked for normality and homoscedasticity by plotting fitted values against residuals and data were log-transformed where necessary.

Three multiple regression models with the explanatory variables Block, Treatment, and average AMF root colonisation during the whole experiment were fitted to assess the contribution of the inoculation treatments and AMF root colonisation to the observed effects on plant N- and P-uptake and biomass and to allow inferences about the mechanisms behind our results. All analyses were performed with the R-statistical software, version 3.0.1.

Results

Overall effect

The MANOVA showed strong treatment effects on plant performance and nutrient leaching for the whole experiment, as well as for year 1 and 2 separately (Table S4). In the following sections, we present the results of univariate two-way ANOVAS for all measured variables for the different years and the whole experiment.

Year 1

Leaching

Leaching losses of NO_3-N, DON, and of total dissolved N were significantly reduced in the ENR-treatment compared to the RED-treatment (Fig. 2, Table S2). In the first year, total N leached was 74.4 kg ha^{-1} for the ENR-treatment and 150.6 kg ha^{-1} for the RED-treatment. This is a reduction of total N leaching by 51.5 % corresponding to an amount of 76.2 kg N ha^{-1}. Approximately 86 % of total N leaching occurred in the form of NO_3-N and on average 13.5 % was in dissolved organic form (Figure 2, Table S2).

In contrast, total P and reactive P leaching was significantly higher in the ENR-treatment, with total P leached amounting to 0.2 kg ha^{-1} for the ENR- and 0.12 kg ha^{-1} for the RED-treatment. Leaching of

unreactive P was significantly reduced in the ENR-treatment (Fig. 3, Table S2). SO_4 leaching was also significantly reduced in the ENR treatment, amounting to 108.7 kg ha^{-1} in the ENR- and 128.1 kg ha^{-1} in the RED-treatment (Table S2).

Crop performance

Maize yield was 40.3 t ha^{-1} in the ENR- and 33.2 t ha^{-1} in the RED-treatment, corresponding to an increase of 22.3 % in the ENR-treatment (Fig. 4a). ENR plants also contained 28.9 % more N and 110 % more P than RED plants (Fig. 4b; Table S2). The grass-mixture grown as intercrop under the maize performed slightly better in the RED-treatment compared to the ENR-treatment, but its biomass was much lower than the maize biomass (Table S2).

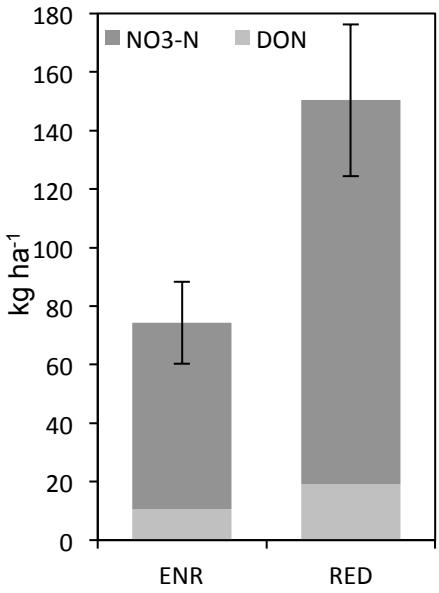

Figure 2: Leaching losses of DON and NO_3^--N in lysimeters inoculated with an enriched (ENR)- or reduced (RED) soil-life inoculum- in year 1 of the experiment. Error bars show ± 1 standard error of total N leaching losses (n=8). Note that NH_4 leaching was very low compared to the other N compounds leached (Soil biota: 0.18 kg NH_4-N ha^{-1}, Control: 0.21 kg NH_4-N ha^{-1}), and is, hence, not displayed here. DON, dissolved organic N

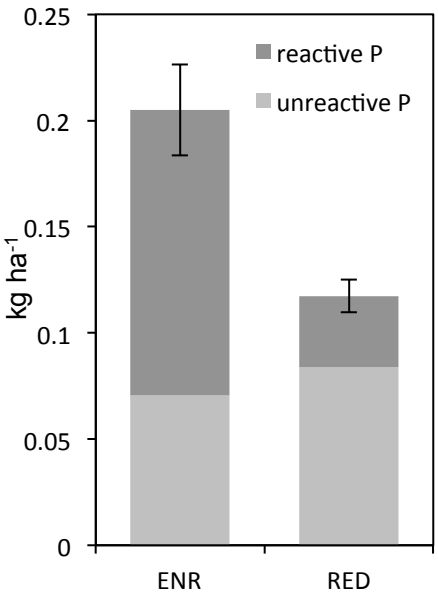

Figure 3: Leaching losses of different P fractions from lysimeters inoculated with an enriched (ENR)- or reduced (RED) soil-life inoculum in year 1 of the experiment. Error bars show ± 1 standard error of total P leaching losses (n=8)

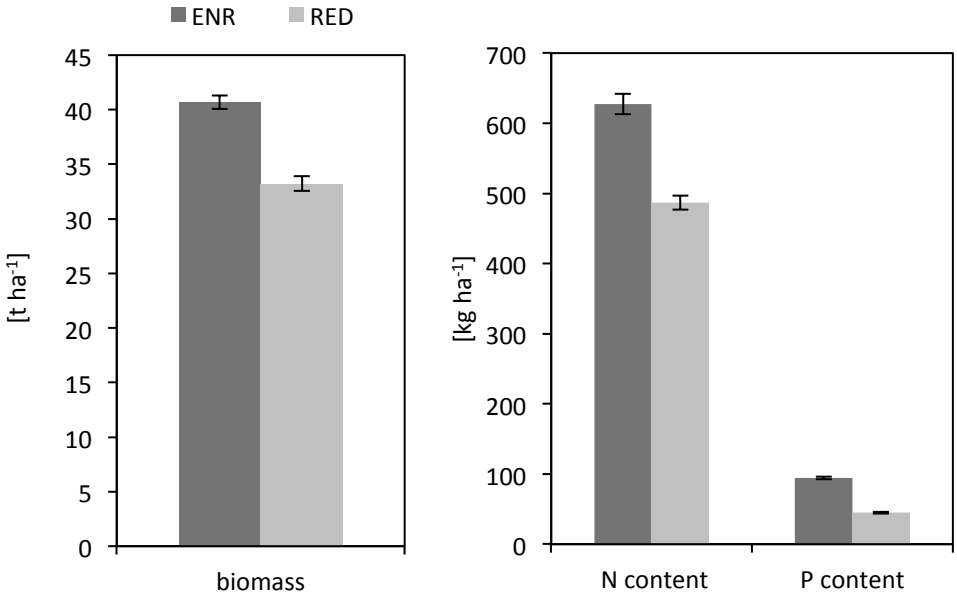

Figure 4: Maize biomass (a) and nutrient contents (b) of plants grown in lysimeters inoculated with an enriched (ENR, dark grey) - or reduced soil-life inoculum (RED, light grey). Error bars show ± 1 standard error (n=8)

Year 2

Leaching

In the second year, the differences in leaching losses between the treatments were smaller and not significant. Total N leached averaged 109.9 kg ha^{-1} in the ENR- and 93.0 kg ha^{-1}in the RED-treatment. Total P leached amounted to 0.3 and 0.27 kg ha^{-1} in the ENR- and RED-treatment, respectively. Leaching of unreactive P was significantly reduced in the ENR-treatment, compared to the RED-treatment. The amount of SO_4 leached was lower than in the first year and was significantly reduced in the ENR-treatment (Table 3).

Crop performance

Total wheat biomass, P content, and P concentration were significantly increased in the ENR- compared to the RED-treatment, with only a slight increase in N content (Table 3). Wheat yield did not differ significantly between the treatments, but P concentration in the grains was significantly increased by 33.3 % in the ENR-treatment. Biomass of weeds growing with the wheat was also higher in the ENR- compared to the RED-treatment (Table 3). In the grass-clover mixture sown after wheat, no difference in plant biomass between the treatments was detected (Table 3).

Table 3: Leaching losses, plant biomass, nutrient contents and the respective ANOVA results for lysimeters inoculated with an enriched (ENR)- or reduced (RED) soil-life inoculum in year 2. Means are shown ± 1 standard error (n=8)

leaching losses	ENR		RED		*df*	*F-value*	*P-value*
NO_3^--N [kg ha^{-1}]	96.48	(9.47)	78.7	(5.94)	1,13	2.44	0.142
NH_4-N [kg ha^{-1}]	0.65	(0.01)	0.71	(0.02)	1,13	4.12	0.063
DON [kg ha^{-1}]	12.72	(0.89)	13.52	(0.37)	1,13	0.68	0.426
TDN [kg ha^{-1}]	109.9	(10.24)	93.0	(6.24)	1,13	1.93	0.189
reactive P [kg ha^{-1}]	0.23	(0.02)	0.19	(0.03)	1,13	1.29	0.276
unreactive P [kg ha^{-1}]	0.07	(0.00)	0.08	(0.00)	1,13	6.63	0.023
Total P [kg ha^{-1}]	0.30	(0.02)	0.27	(0.03)	1,13	0.55	0.473
SO_4^{2-} [kg ha^{-1}]	62.8	(3.05)	76.1	(3.17)	1,13	8.44	0.012
Plant biomass and nutrient data							
Wheat							
biomass							
Total [t ha^{-1}]	10.6	(0.20)	9.1	(0.52)	1,13	7.22	0.019
N content [kg ha^{-1}]	154.1	(4.58)	140.5	(5.93)	1,13	3.2	0.098
P content [kg ha^{-1}]	35.96	(1.16)	19.9	(2.26)	1,13	37.0	0.000
N concentration [mg kg DW^{-1}]	14.52	(0.30)	15.65	(0.64)	1,13	2.75	0.121
P concentration [mg kg DW^{-1}]	3.38	(0.06)	2.24	(0.28)	1,13	15.1	0.002
N/P-ratio*	4.29	(0.07)	7.57	(0.72)	1,13	27.2	0.000
Yield (kernels)							
total [t ha^{-1}]	3.40	(0.08)	3.50	(0.07)	1,13	2.93	0.110
N content [kg ha^{-1}]	104.5	(2.26)	106.4	(2.45)	1,13	0.32	0.580
P content[kg ha^{-1}]	20.29	(0.65)	15.79	(1.40)	1,13	8.44	0.012
N concentration [mg kg DW^{-1}]	31.04	(0.36)	30.47	(0.71)	1,13	0.75	0.402
P concentration [mg kg DW^{-1}]	6.02	(0.09)	4.51	(0.38)	1,13	13.81	0.003
weed biomass [t ha^{-1}]	0.49	(0.13)	0.19	(0.04)	1,13	4.67	0.050
Grassclover							
Total [t ha^{-1}]	1.27	(0.25)	1.17	(0.12)	*1,13*	*0.14*	*0.711*
N content [kg ha^{-1}]	35.86	(5.47)	32.6	(2.73)	1,13	0.30	0.596
P content [kg ha^{-1}]	4.44	(0.92)	3.77	(0.41)	1,13	0.48	0.501
N concentration [mg kg DW^{-1}]	29.82	(1.30)	28.11	(0.74)	1,13	1.51	0.241
P concentration [mg kg DW^{-1}]	3.38	(0.15)	3.2	(0.07)	1,13	1.34	0.267
N/P-ratio	8.98	(0.65)	8.81	(0.35)	1,13	0.07	0.790
**log transformed*							

DON, dissolved organic N; TDN, total dissolved N

Cumulative nutrient loss and plant uptake for the whole experiment

The cumulative amount of N lost through leaching was 184.3 kg ha^{-1} in the ENR- and 243.6 kg ha^{-1} in the RED-treatment. This is a reduction of 24.3 % in the ENR-treatment, corresponding to 59.3 kg N ha^{-1} (Fig. 5a). Total N in plant biomass was 879.9 kg N ha^{-1} in the ENR-treatment and 747.2 kg N ha^{-1} in the RED-treatment. This is an increase of 17.8 % in the ENR-treatment, corresponding to 132.7 kg N ha^{-1} (Fig. 5a). Over the whole experiment, relative N leaching, defined as kg N leached per kg N plant uptake, amounted to 0.21 kg N in the ENR-treatment and 0.33 kg N in the RED-treatment (Table S3). Total P leached amounted to 0.51 kg ha^{-1} and 0.39 kg ha^{-1} in the ENR- and RED-treatment, respectively. This is an increase of 0.12 kg ha^{-1} in the ENR-treatment (Fig. 5b). The fraction of unreactive P leaching was, however, significantly reduced in the ENR-treatment (Table S3).

Total P in plant biomass amounted to 121.8 kg ha^{-1} and 70.7 kg ha^{-1} in the ENR- and RED-treatment, respectively. This is an increase of 72.3 %, corresponding to 51.1 kg P ha^{-1} (Fig. 5b). Relative P leaching (g P leached/ kg P plant uptake) was lower in the ENR treatment and amounted to 4.13 g P, while in the RED treatment relative P leaching was 5.47 g P (Table S3). 28 % of P lost was in the form of unreactive P.

Total leaching losses of SO_4 amounted to 171.5 kg ha^{-1} and 204.2 kg ha^{-1} in the ENR- and RED-treatment, respectively. This is a reduction of 16 % in the ENR-treatment, corresponding to 32.7 kg ha^{-1} (Table S3). The total amount of leachate did not differ significantly between the treatments (P=0.08).

AMF root colonization and microbial biomass

Four months after the start of the experiment, roots of the RED-treatment already showed 10.25 % root colonization by AMF, while the ENR-treatment showed 75 % root colonization (Table 4). The AMF root colonization of wheat was higher compared to maize in both treatments. The ENR-treatment had 88.6 % of root length colonized, while the RED-treatment had 28.1 % root length

colonized. In the grass-clover mixture, the root colonization in the ENR-treatment increased further, and the difference between the treatments decreased, although remaining significant (Table 4). Microbial biomass C and N contents did not differ significantly between the treatments in both years (Table 4).

Table 4: AMF colonization measures of the different crops, microbial biomass C and N contents and the respective statistical test results for lysimeters inoculated with an enriched (ENR)- or reduced (RED) soil-life inoculum. Means are shown ± 1 standard error (n=8)

		ENR		RED		*df*	χ2	*P-value*
*AMF root colonization (%)**								
maize/grass								
	Total	70.8	(2.55)	10.25	(4.73)	1	11.3	0.001
	vesicles	1.63	(0.60)	2.88	(1.47)	1	0.00	0.957
	arbuscles	26	(2.33)	14.38	(3.16)	1	5.85	0.016
wheat								
	Total	88.63	(1.91)	28.13	(9.39)	1	11.33	0.001
	vesicles	15.63	(1.65)	5.13	(2.89)	1	6.47	0.011
	arbuscles	15.8	(2.72)	3.63	(1.57)	1	9.70	0.002
grass-clover								
	Total	75.0	(2.75)	43.0	(8.95)	1	5.84	0.016
	vesicles	6.5	(2.15)	0.63	(0.50)	1	9.14	0.002
	arbuscles	26.13	(3.91)	0.75	(0.41)	1	11.65	0.001
*microbial biomass***								
Year 1						*df*	*F-value*	*P-value*
	MibiC [t ha^{-1}]	1.75	(0.06)	1.62	(0.08)	1,13	2.04	0.177
	MibiN [t ha-1]	0.29	(0.01)	0.26	(0.02)	1,13	1.03	0.328
Year 2								
	MibiC [t ha-1]	1.40	(0.04)	1.44	(0.05)	1,13	0.68	0.425
	MibiN [t ha-1]	0.24	(0.01)	0.24	(0.01)	1,13	0.55	0.472

* non-parametric kruskal-wallis-tests were performed
** ANOVA was performed

Contribution of AMF root colonisation to plant N- and P-uptake

The multiple regression models containing Block, Treatment, and AMF root colonisation as predictors explained 70% of plant biomass, 66% of plant N uptake and 97% of plant P uptake. AMF root colonisation was a significant predictor of plant P uptake, but not of plant N uptake and biomass. The inoculation treatment was a significant predictor of plant P uptake and plant biomass but not of plant N uptake (Table S5).

Soil analyses

At the end of the experiment, available soil P extracted with CO_2-saturated water was significantly reduced by 39.5 % in the ENR-treatment compared to the RED-treatment. For PAAE, similar results were found. However, all other soil parameters did not differ significantly between the treatments. P export from soil through plant uptake and leaching was higher than the decline in PAAE during the course of the experiment. We calculated the difference between P removed from the AAE-extractable soil P-pool and P exported through plant uptake and leaching (P_{mob}). This gives an indication of how much soil P was mobilized from non-AAE extractable soil pools during the course of the experiment. In the ENR-treatment, an additional 62.8 kg P ha^{-1} had been mobilized, while in the RED-treatment only an additional 30.5 kg P ha^{-1} been mobilized from soil. Therefore, the presence of soil biota increased P mobilization from initially non-available soil P resources by 118 % (Table 5).

Table 5: Soil parameters in the top soil measured at the end of the experiment and the respective ANOVA results for lysimeters inoculated with an enriched (ENR)- or reduced (RED) soil-life inoculum. Means are shown ± 1 standard error (n=8)

soil parameters	ENR		RED		*df*	*F-value*	*P-value*
Humus [t ha^{-1}]	162.76	(3.81)	156.22	(3.87)	1,13	1.44	0.252
organic C [t ha^{-1}]	94.24	(1.97)	90.46	(2.16)	1,13	1.70	0.215
total N [t ha^{-1}]	11.12	(0.26)	10.90	(0.29)	1,13	0.38	0.549
availP [kg ha^{-1}]	1.78	(0.06)	2.58	(0.09)	1,13	59.54	0.000
P_{AAE} [kg ha^{-1}]	68.59	(2.28)	87.59	(3.98)	1,13	19.64	0.001
K_{AAE} [kg ha^{-1}]	606.6	(6.90)	606.9	(15.70)	1,13	0.00	0.989
Mg_{AAE} [t ha^{-1}]	10.48	(0.13)	10.13	(0.15)	1,13	2.94	0.110
Ca_{AAE} [t ha^{-1}]	117.03	(3.07)	118.1	(5.91)	1,13	0.03	0.866
pH (H_2O)	7.96	(0.02)	7.95	(0.02)	1,13	0.35	0.564
P_{mob} [kg ha^{-1}]	62.78	(3.16)	30.54	(4.37)	1,13	38.95	0.000

P_{mob}, P mobilized from initially non-AAE-extractable soil P

Discussion

Our study demonstrates that soil-life contributes substantially to agricultural sustainability by supporting plant nutrient uptake and plant yield, and by reducing N leaching losses. This also indicates that soil-life enhanced nutrient use efficiency in our cropping system. Throughout the experiment, N leaching was reduced, while plant N and P contents were strongly increased in lysimeters with enriched soil-life compared to lysimeters with a reduced soil-life treatment. To our knowledge, this is the first study showing that soil biota in the ranging between 11µm and 2mm, including AMF and the soil micro-and mesofauna, can exert strong effects on nutrient leaching and crop performance in an agricultural crop rotation.

Our observation is important because large amounts of N are lost through leaching from croplands (Liu et al., 2010). For instance, in Western-European countries, estimated N leaching losses range from 5 to 102 kg N $ha^{-1}a^{-1}$ (Leip et al., 2008), with losses up to 160 kg N $ha^{-1}a^{-1}$being reported (Herzog

et al., 2008). Commonly, the highest leaching losses occur in areas under intensive agriculture, where soil diversity and the abundance of AMF is often reduced (de Vries et al., 2013; Verbruggen et al., 2010). Hence, our results indicate that agricultural sustainability, and in particular nitrogen use efficiency, could be enhanced by management practices that support soil-life such as reduced tillage, crop rotation, or mulching (Giller et al., 1997; Wardle, 1995).

In the ENR-treatment, the soil P pools were significantly reduced and the amount of additional P mobilized from non-AAE extractable soil-pools increased more than two-fold (Table 5). The observed increase in total phosphorus leaching is, thus, likely to be a by-product of enhanced mineralization and mobilization of soil P resources by soil biota. However, the amounts of P lost during a period of almost 2 years were small, and the difference, even if significant, between the ENR- and the RED-treatment was only 116g ha^{-1}. When considered in relation to the increase in total plant P uptake of more than 51 kg ha^{-1}, this amount of P appears negligible. The relative P losses compared to the amount of P taken up by the plant biomass were significantly lower in the ENR-treatment than in the RED-treatment, indicating that the P use efficiency was increased in the presence of an enriched soil-life. The dimensions of total plant N and P uptake and total N and P leaching are visualized in Figure 5.

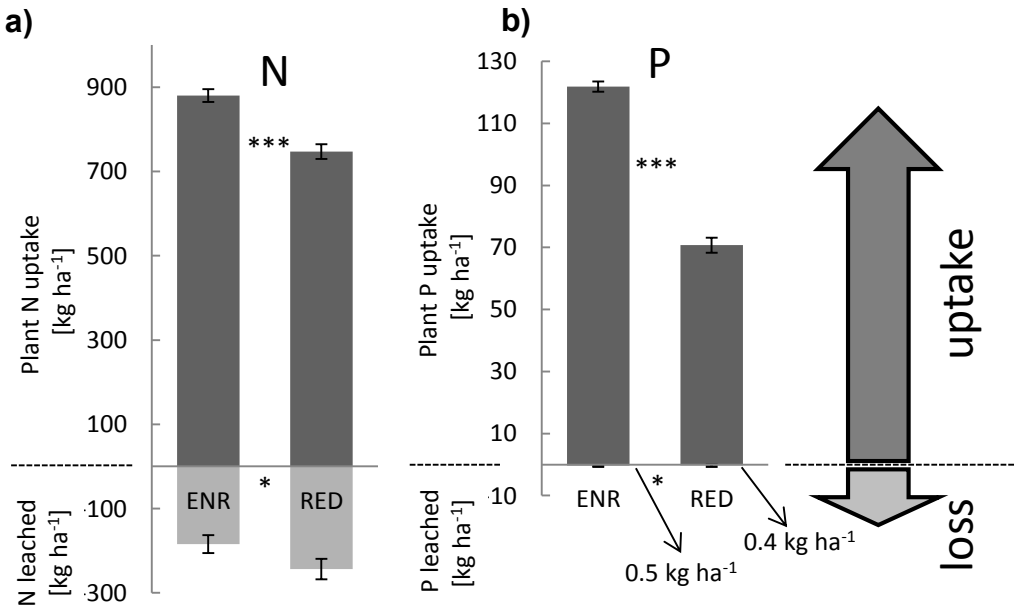

Figure 5: Cumulative plant N uptake and N leaching (a) and plant P uptake and leaching (b) of plants grown in lysimeters inoculated with an enriched (ENR)- or reduced (RED) soil-life inoculum during the whole experimental period. Error bars show ± 1 standard error (n=8). Significant differences between the ENR- or RED-treatment are indicated by asteriscs (***, P<0.001;**, P <0.01; *, P<0.05)

Enriched soil-life significantly increased maize and wheat biomass and P and N contents, as well as P concentrations. These results imply that soil biota have the potential to improve the quantity and quality of agricultural yields. Plant N concentrations were, however, not affected. Plant N:P ratios have been proposed as a tool to assess nutrient limitation of plant communities (Koerselman and Meuleman, 1996). The N:P ratios in maize and wheat plant tissue were all below 14, indicating N limitation, but they were significantly lower in the ENR-treatment (Tables 2 & S2). This was mainly driven by a strong increase in plant P uptake in the ENR-treatment. The multiple regression models indicate that AMF played an important role in increased plant P nutrition, while no direct effects of AMF root colonisation on plant N uptake and biomass were observed. Hence, by significantly improving P nutrition, AMF probably increased plant N limitation and, thus, created an N drain towards plant biomass, providing an explanation for the reduction in N leaching.

It has also been shown that AMF can enhance N interception from soil, store substantial amounts of N in their extraradical hyphae, and transport N to plants (Hodge and Fitter, 2010; Johansen et al., 1992), which could provide an additional mechanism for reduced N leaching. However, the results of this study do not reveal a direct effect of AMF on N cycling. The inoculation treatment effect also significantly explained a portion of plant P uptake, as well as of plant biomass in the multiple regression. This indicates that factors other than AMF root colonisation contributed to the results.

On average, 107 kg N ha^{-1} was leached from soil per year. This is at the upper limit of estimated N-leaching losses for many European countries (Leip et al., 2008). P leaching losses were low and comparable to other studies analysing P leaching losses in agricultural systems (Neumann et al., 2012; Ulen, 1999). The relatively high N leaching losses could be attributed to enhanced nutrient availability through soil sterilization (McNamara et al., 2003) and enhanced nutrient release from organic matter mineralization due to a higher soil temperature in the lysimeters (Kirschbaum, 1995). In a Swiss lysimeter experiment using non-sterile soil, comparable amounts of leaching losses were reported (Spiess et al., 2011).

Maize yield was higher compared to yields commonly achieved in Swiss agriculture (Dubois et al., 1995; Rüegg et al., 1998). Enhanced nutrient availability in the lysimeters and "edge effects" such as reduced competition from neighbouring plants for nutrients and light compared to field conditions probably contributed to the high yields.

The average root length colonized by AMF during the whole experiment amounted to 78% in the ENR-treatment and 27% in the RED-treatment. The root colonisation levels in the RED-treatment were comparable to Swiss fields under conventional management, while the root colonisation in the ENR-treatment was considerably higher than values found in Swiss organic fields (40-50% of root length colonised, Honegger et al. (2014)). However, AMF root colonisation in the RED-treatment reached values of 40 % root length colonized in the second year, a value comparable to colonization levels in Swiss organic fields.

Wind borne contamination from soil particles, microbes from neighbouring fields, and rain splash are the most likely causes for the increase in AMF abundance in the RED-treatment. Increased import of AMF and micro- and mesofauna to the RED-treatment might also explain the less pronounced differences between the treatments in the second year.

The ENR-treatment consisted of soil biota with a size of ≤2 mm, while the RED-treatment received soil microorganisms passing through an 11 µm filter, including some protozoa. The effects shown here must have been induced by soil organisms ranging between 11 µm and 2 mm in size, i.e. meso- and microfauna and AMF. Earlier studies showed that soil mesofauna, comprised of organisms like collembola and mites, can break down organic matter and release mineral nutrients into soil (Bardgett and Chan, 1999; Brussaard et al., 1995). Members of soil microfauna, including fungal, bacterial, or root feeding protozoa and nematodes, can increase nutrient mineralisation, making nutrients available to plants (Griffiths, 1986; Woods et al., 1982). AMF most likely played an important role in recycling nutrients released into the soil by enhancing nutrient interception and nutrient transfer to the plants. Furthermore, effects due to changes in plant physiology, or differences in the microbial communities arising from different ecological processes and different foodwebs developing within the inoculums and treatments, could have had an influence. With the approach employed in this study, it was not possible to precisely identify the organisms, interactions, and processes responsible for the effects on plant yield and nutrient cycling. Future work should manipulate specific functional groups to elucidate specific mechanisms.

Several studies report that soil-life can be enhanced by management practices that increase the organic matter content of soils and reduce soil disturbance. For example collembolans, mites, and nematodes were shown to be favoured by strip-tilling combined with cover cropping (Wang et al., 2011), and the abundance or biomass of protozoa and nematodes are often found to be increased in organically managed soils compared to conventionally managed soils (Foissner, 1992). Additionally, management practices like stubble retention and reduced tillage were shown to increase the abundance of protozoa, nematodes, collembolan, and mites (Roper and Gupta, 1995). Reduced

tillage and reduced fertiliser inputs, especially for P, are also often reported to promote the abundance of AMF (Helgason et al., 1998; Kahiluoto et al., 2000) and the ability of AMF to support plant P uptake (Köhl et al., 2014). In the most intensively managed fields in the Netherlands, AMF were absent or nearly absent (Verbruggen et al. 2010). Such conditions might be comparable to our RED-treatment in the first year of the experiment where AMF abundance was low and where we observed very high nutrient losses.

In a large scale correlative field study by de Vries et al. (2013), it was reported that AMF may contribute to reduced N leaching in agricultural land-use systems. Another study (Wagg et al., 2014) showed that a decline in soil biodiversity can negatively affect several ecosystem functions. These results are in line with the results presented here, and indicate that high nutrient losses in intensively managed fields may partly result from the disruption of soil food webs. Further work should now specifically test whether agricultural sustainability and nutrient use efficiency is higher in agricultural fields with enriched soil-life.

P fertilizers are often applied in excess, because a large fraction of the applied P quickly reacts with the soil environment rendering it unavailable to plants (Barberis et al., 1995). Our results demonstrate that in the presence of soil biota, soil P resources normally unavailable to plants can efficiently be mobilized. Therefore, P fertilization could be reduced, sparing globally limited P resources.

In conclusion, this study demonstrates that the implementation of agricultural management practices that promote soil-life may enhance agricultural sustainability by promoting plant nutrient uptake and reducing nutrient leaching losses, resulting in greater nutrient use efficiency of agricultural ecosystems. The effects that different agricultural management practices exert on soil biota must be seriously taken into account when aiming to enhance the sustainability of cropping systems.

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Supporting Information

Appendix S1: Supporting methods

Soil processing and sterilization

The soil was processed with an earth crusher to remove big stones (>2 cm) and to crush big soil clumps. Afterwards, the soil was filled into boxes and sealed with polypropylene-foil. Boxes containing soil were then sterilized with x-ray radiation with a dose range of 8 to 25 kGy. This radiation dose serves to eliminate most soil biota, including AM fungi and invertebrates, but it does not lead to complete soil sterilization as some microbes might survive (McNamara et al., 2003).

Inoculum production

Fresh field soil was taken from the same pasture used to fill the lysimeters and was sieved twice to 2 mm to remove any macrofauna, stones, and large organic debris. Half of the sieved soil was then autoclaved (120 °C, 90 min). A total of 32 pots were set up for inoculum preparation. Per pot, 6 L of an autoclaved 17:3 sand:soil-mixture was mixed with either 600ml of the sieved fresh field soil or 600ml of autoclaved field soil. 70 ml of a microbial wash was added to each pot (Koide and Li, 1989). The microbial wash served to introduce a similar set of soil microorganisms to both inoculums and to balance out differences in the soil microbial communities between the fresh and the autoclaved field soil. It was produced by suspending 700 g fresh field soil in 3 L of water and subsequent filtering through a Whatman no. 1 filter paper (Whatman Ltd., Springfield Mill, UK) with a pore size of 11 µm. The filtrate did not contain any AM fungal propagules, only smaller microorganisms. Pots were then planted with *Plantago lanceolata L.* and grown in the greenhouse for 4 months with regular watering and fertilization (see Table S1 for details).

After 16 weeks, the pots were watered for the last time and slowly dried before harvesting. The above ground plant biomass was removed, soil substrate collected from the pots, and the roots were cut into pieces. This mixture of soil and roots pieces served as the two treatment inocula in the lysimeters.

Lysimeter filling

Before filling the lysimeters with soil, 22 L of a sterilized mixture of sand and fine gravel was added to the bottom of the lysimeters to a height of 8 cm in order to improve drainage. Three weeks after sterilization, 90 L of subsoil was filled into each lysimeter, making up approx. 33cm of the soil column. The subsoil was compacted by jumping on it repeatedly. A total of 90 L of topsoil and 13 L of the appropriate treatment inoculum was then added into each lysimeter in 12 alternating layers, making up approx. 35 cm of the soil column. Additionally, 700 ml of a microbial wash was produced from 280 g of fresh field soil and 100 g of each of the two inoculums using the same methods described above. This wash was homogeneously added to the topsoil of each lysimeter to minimize unintended differences in soil microbial communities between the treatments.

Finally, an additional 12 L of sterilized, uninoculated topsoil was added to the top of the lysimeters to avoid cross contamination. Transparent PET-foil was attached between the edge of the lysimeter and soil column, encompassing the lysimeter and looming approx. 40 cm into the air. This served to protect the lysimeters against air borne contamination from neighbouring lysimeters and rain splash from the adjacent ground (see Figs.1 & S1).

The lysimeters were filled on 20 and 21/04/2011, covered with plastic foil, and left unplanted for three weeks to allow stabilization of soil chemical properties before starting the experiment. Before planting, the lysimeters were flushed with 30 L of water to induce leaching and remove excess nutrients resulting from sterilization.

Planting

On 10/05/2011, 9 seeds of Maize (*Zea mais*, var. PR 39 G12 BIO, Pioneer Inc., IA, USA) were planted into each lysimeter. After germination, plants were thinned to 3 per lysimeter.

On 07/07/2011, 1 g of a grass seed mixture containing 50 % *Lolium perenne L.*, var. Arara, 35 % *Festuca pratensis L.*, var. Praxilla and 15 % *Lolium multiflorum L.*, var. Oryx was sown between the maize plants. This was done to provide AMF host plants over the winter after the maize harvest. The

maize plants developed relatively fast in the lysimeters and were harvested on 31/08/ 2011. The grass mixture grew until it was harvested on 10/03/2012.

On 15 and 16/03/2012 the lysimeters were superficially ploughed (0-5 cm) with a hand rake. Large maize and grass residues were removed to assure homogeneous conditions in the lysimeters. Afterwards, summer wheat, *Triticum aestivum* L., var. Fiorina, was sown and was harvested on 31/07/2012. Immediately following the wheat harvest, the lysimeters were ploughed again with a hand rake, and a grass-clover mixture (standard mixture 330; Suter et al., 2012) was sown and grew until the end of the experiment in January 2013.

Sampling

Leachate samples were collected bi-weekly, or when the containers were full. In the summer months, which are often accompanied by dry periods and high evapotranspiration, no leaching occurred. Each of the leachate samples were weighed, mixed thoroughly by shaking, and a 50 ml subsample was taken for nutrient analyses. A total of 456 leachate samples were analysed throughout the experiment.

In order to assess root colonization by AMF, root samples were taken from maize shortly before the harvest on 28/08/2011, from wheat on 24/07/2012, and from the grass-clover mixture on 12/11/2012. In order to determine soil microbial biomass C and N, soil samples were taken on 28/08/2011 and on 12/11/2012 with a core sampler. Soil samples for nutrient analyses were also taken on 12/11/ 2012. All plants were harvested by cutting them approx. 5 cm above the soil surface. Maize and wheat plants were air dried, and maize kernels were removed from the cob by hand. The wheat was threshed with a threshing machine. Maize and wheat kernels were weighed and ground in a centrifuge mill (0.25 mm). Corncobs and remaining maize biomass were mechanically shredded. Grass and grass-clover biomass was dried at 60 °C and maize, wheat, grass and grass-clover biomass was ground in a cutting mill for nutrient analyses.

Weeds growing under the wheat were harvested separately, dried at 60 °C, and weighed. Because weed biomass made up less than 5 % of total biomass, it was not analysed for its nutrient concentrations.

Leachate analyses

The leachate flowing through the 8 cm sand-gravel mixture in the bottom of the lysimeters was clear and therefore was not filtered before analyses. Concentrations of NO_3-N, NO_2-N, PO_4-P, and SO_4 were determined using a Dionex DX500 anion chromatograph (Dionex Corporation, Sunnyvale, CA, USA).

Total P content in the leachate was determined using Oxisolv® (Merck, Darmstadt, DE) oxidation prior to photometric analysis with a spectrophotometer (Helios Gamma, Thermo Scientific, Digitana AG, Switzerland) according to Watanabe and Olsen (1965). NH_4-N was analysed using a Skalar segmented flow analyser (Skalar, Breda, NL) according to the reference methods of the Swiss Federal Research Stations (Eidgenössische Forschungsanstalten FAL et al., 1996).

Total dissolved N (TDN) was measured by chemoluminescence (DIMATOC® 2000 coupled with a DIMA-N analyser, Dimatec, Essen, DE). Amounts of NO_2-N were low and were added to the NO_3-N values. The difference between TDN and mineral N (NO_3-N and NH_4-N) was considered as dissolved organic N (DON).

The amount of PO_4-P in the samples was labelled reactive P. The difference between total P and reactive P was labelled unreactive P. This fraction comprises all compounds not directly available to plants such as soluble and particulate organic P compounds, polyphosphates, and particulate inorganic material, e.g. clays (Daniel and DeLaune, 2009).

All nutrient concentrations were multiplied with the leachate volume to calculate the total amount of nutrients lost per lysimeter. Leaching data is presented on a yearly basis with year 1 ranging from maize sowing in May 2011 until harvest of the grass-mixture in March 2012, and year 2 ranging from wheat sowing in March 2012 to grass-clover harvest in January 2013.

AM fungal root infection

The percentage of root length colonized by AMF was determined from root samples stored in 50% ethanol after staining with pen ink (Vierheilig et al., 1998) using a modified line-intersection method for 100 intersections per sample (McGonigle et al., 1990).

Soil microbial biomass C and N

Microbial biomass C and N estimates by chloroform-fumigation-extraction were carried out on duplicate samples according to Vance et al. (1987). Organic C (TOC) in the extracts was determined by infrared spectrometry after combustion at 850°C (DIMATOC® 2000, Dimatec, Essen, Germany). Total N was subsequently measured in the same sample by chemoluminescence (TNb, Dimatec, Essen, DE). Microbial biomass C and N was calculated according to Jörgensen (1996) and Jörgensen and Mueller (1996).

Figure S1: Maize plants growing in the lysimeters in the first year of the experiment in July 2011, around 1 month before harvest. Note the rich and diverse plant community adjacent to the lysimeters, probably serving as an inoculum source leading to the contamination of the Reduced soil-life lysimeters with soil biota during the course of the experiment.

Table S1: Composition of the nutrient solution used for inoculum production. Every 4 weeks, each pot received 30 ml of the solution

Compound	Concentration
KNO_3	10 mM
$(NH_4)_2SO_4$	5 mM
$MgSO_4$	1 mM
KH_2PO_4	1.5 mM
$CaCl_2$	2 mM
KCl	50 µM
H_3BO_3	25 µM
$MnSO_4$	2 µM
$ZnSO_4$	2 µM
$CuSO_4$	0.5 µM
Na_2MoO_4	0.5 µM
Fe-(Na)EDTA	20 µM

Table S2: Leaching losses, plant biomass, nutrient contents and the respective ANOVA results for lysimeters inoculated with an enriched soil-life (ENR)- or a reduced soil life (RED)- inoculum in year 1. Means are shown ±1 standard error (n=8)

leaching losses	ENR		RED		*df*	*F-value*	*P-value*
NO_3^--N [kg ha-1]*	63.67	(12.87)	131.3	(23.90)	1,13	9.80	0.008
NH_4^+-N [kg ha-1]	0.18	(0.01)	0.21	(0.02)	1,13	2.78	0.119
DON [kg ha-1]	10.57	(1.41)	19.08	(2.17)	1,13	13.01	0.003
TDN [kg ha-1]	74.42	(13.96)	150.6	(25.98)	1,13	10.68	0.006
reactive P [kg ha^{-1}]	0.13	(0.02)	0.03	(0.01)	1,13	32.54	0.000
unreactive P [kg ha^{-1}]	0.07	(0.00)	0.08	(0.00)	1,13	5.34	0.038
total P [kg ha^{-1}]	0.20	(0.02)	0.12	(0.01)	1,13	14.95	0.002
SO_4^{2-} [kg ha^{-1}]	108.7	(5.66)	128.1	(6.33)	1,13	5.90	0.030
Plant biomass and nutrient data							
Maize							
Biomass							
Total [t ha^{-1}]	40.7	(0.64)	33.2	(0.67)	1,13	92.74	0.000
N content [kg ha^{-1}]	627.3	(14.31)	486.7	(9.81)	1,13	105.3	0.000
P content [kg ha^{-1}]	94.34	(2.01)	44.9	(1.26)	1,13	442.6	0.000
N concentration [g kg DW^{-1}]	15.42	(0.30)	14.66	(0.24)	1,13	3.78	0.074
P concentration [g kg DW^{-1}]	2.32	(0.04)	1.35	(0.04)	1,13	309.3	0.000
N/P-ratio	6.65	(0.07)	10.9	(0.26)	1,13	293.4	0.000
Grass							
Total [t ha^{-1}]	2.04	(0.28)	2.76	(0.41)	1,13	3.57	0.081
N content [kg ha^{-1}]	62.63	(8.14)	87.44	(12.48)	1,13	4.90	0.045
P content [kg ha^{-1}]	7.23	(0.99)	10.03	(1.28)	1,13	4.69	0.050
N concentration [g kg DW^{-1}]	31.14	(1.04)	32.28	(1.10)	1,13	0.53	0.478
P concentration [g kg DW^{-1}]	3.54	(0.06)	3.79	(0.16)	1,13	2.72	0.123
N/P-ratio	8.78	(0.23)	8.56	(0.27)	1,13	0.43	0.526
*log transformed							

DON, dissolved organic N; TDN, total dissolved N

Table S3: Cumulative leaching losses, plant biomass, nutrient contents and relative leaching related to plant uptake and the respective ANOVA results for lysimeters inoculated with an enriched soil-life (ENR)- or a reduced soil-life (RED)-inoculum for the whole experimental period (year 1 and 2 added). Means are shown ±1 standard error (n=8)

Leaching	ENR		RED		*df*	*F-value*	*P-value*
NO_3^--N [kg ha^{-1}]	160.2	(19.39)	210	(22.45)	1,13	4.07	0.065
NH_4^+-N [kg ha^{-1}]	0.84	(0.01)	0.93	(0.02)	1,13	10.04	0.007
DON [kg ha^{-1}]	23.29	(2.03)	32.6	(2.22)	1,13	11.74	0.005
TDN [kg ha^{-1}]	184.3	(21.13)	243.6	(24.54)	1,13	4.78	0.048
reactive P [kg ha^{-1}]	0.36	(0.02)	0.22	(0.03)	1,13	11.72	0.005
unreactive P [kg ha^{-1}]	0.14	(0.01)	0.17	(0.00)	1,13	12.75	0.003
total P [kg ha^{-1}]	0.5	(0.03)	0.39	(0.04)	1,13	6.28	0.026
SO_4^{2-}	171.5	(7.20)	204.1	(6.06)	1,13	13.24	0.003
Biomass							
Total [t ha^{-1}]	55.1	(0.67)	46.4	(1.13)	1,13	40.73	0.000
N content [kg ha^{-1}]	879.9	(15.03)	747.2	(17.38)	1,13	31.27	0.000
P content [kg ha^{-1}]	121.9	(1.68)	70.73	(2.45)	1,13	276	0.000
N_{rel} [kg]	0.21	(0.02)	0.33	(0.04)	1,13	9.2	0.010
P_{rel} [g]	4.12	(0.20)	5.45	(0.43)	1,13	7.44	0.017

DON, dissolved organic N; TDN total dissolved N
N_{rel}, relative N leaching (kgN leached/ kgN plant uptake)
P_{rel}, relative P leaching (gP leached/ kgP plant uptake)

Table S4: MANOVA output investigating effects of Block and Inoculation treatment on nutrient leaching and plant performance for Year 1 (a), Year 2 (b) and the complete experiment (c). Approximate F-values were generated using Pillai`s criterion. Dependent variables were Total plant biomass, Plant N- and P- contents, Total N- and P-leaching and SO_4^{2-}-leaching.

a)
Year 1

	Df	Pillai	*F*-value	num *df*	den *df*	*P*-value
Block	1	0.762	4.261	6	8	0.032
Treatment	1	0.988	110.82	6	8	<0.0001
Residuals	13					

b)
Year 2

	Df	Pillai	*F*-value	num *df*	den *df*	*P*-value
Block	1	0.603	2.025	6	8	0.175
Treatment	1	0.937	19.94	6	8	<0.001
Residuals	13					

c)
complete experiment

	Df	Pillai	*F*-value	num *df*	den *df*	*P*-value
Block	1	0.649	2.470	6	8	0.118
Treatment	1	0.989	122.22	6	8	<0.0001
Residuals	13					

Table S5: Results of multiple regressions explaining Total plant P content (a), Total plant N content (b) and Total plant biomass for the whole experiment with Block, inoculation Treatment and AMF root colonization as predictors. AMF, arbuscular mycorrhizal fungi

a)

response	**Total Plant P**			
	df	F-ratio	p-Value	Adjusted R^2
Regression	3	144.6	0.000	0.9664
Residual	12			

Variable	Coefficient	SE	*t*-ratio	*P*-Value
(Intercept)	98.3914	8.6037	11.436	<0.0001
Block	-0.3344	2.4777	-0.135	0.895
Treatment RED	-35.6956	5.9741	-5.975	<0.0001
mean AMF root colonisation	0.3025	0.1066	2.838	0.015

b)

response	**Total Plant N**			
	df	F-ratio	p-Value	Adjusted R^2
Regression	3	10.66	0.001	0.659
Residual	12			

Variable	Coefficient	SE	*t*-ratio	*P*-Value
(Intercept)	800.341	82.78	9.668	<0.0001
Block	8.755	23.838	0.367	0.72
Treatment RED	-83.618	57.479	-1.455	0.171
mean AMF root colonisation	0.962	1.026	0.938	0.367

c)

response	**Total Plant biomass**			
	df	F-ratio	p-Value	Adjusted R^2
Regression	3	12.78	0.000	0.702
Residual	12			

Variable	Coefficient	SE	*t*-ratio	*P*-Value
(Intercept)	536.2988	48.8206	10.985	<0.0001
Block	-5.8106	14.0591	-0.413	0.687
Treatment RED	-75.2098	33.8991	-2.219	0.047
mean AMF root colonisation	0.2252	0.6048	0.372	0.716

General Discussion

Agriculture is providing the nutritional basis for the global human population but is also a main contributor to environmental pollution. To increase resource use efficiency is a major aspect to enhance the sustainability of cropping systems, to spare limited global resources, alleviating pressures on the environment and to assure food security in the long term.

Soil organisms are key for nutrient cycling and nutrient availability to crops. Many agricultural practices as performed today are, however, detrimental to soil-food webs and rely on external resource inputs to maintain crop yields. These practices assure high agricultural yields but are characterized by high nutrient inefficiency resulting in excessive resource use and environmental hazards. The potential of soil organisms to improve plant nutrition, reduce nutrient losses and enhance the nutrient efficiency and sustainability of plant soil systems is often not considered and knowledge of the potential of soil organisms to improve efficient nutrient cycling is limited. In this PhD thesis, I tried to assess the potential of AMF to improve sustainable nutrient cycling through reducing nutrient losses as gaseous compounds and through leaching and simultaneously improving plant nutrition.

The central questions stated in the introduction were (1) Do AMF affect gaseous emissions of N via denitrification?, (2)Can AMF reduce nutrient leaching from soil?, and (3) Can AMF contribute to sustainable agricultural practices by improving plant nutrition and reducing nutrient losses?

These questions will now be discussed in further detail including reflections on the ecological relevance of the results obtained and the options to exploit the beneficial functional traits of the AMF symbiosis in agriculture .

1) Do AMF affect gaseous emissions of N via denitrification?

In three of the four chapters in this thesis, we collected data on the influence of AM fungi on gaseous emissions of N. We constantly observed a reduction of N_2O fluxes after the application of a

fertilization and water pulse conducive for denitrification. These results were obtained from 3 different soil types with different characteristics and pH values ranging from acid to neutral, suggesting that this effect is rather independent of soil conditions. While in two experiments sterilized and re-inoculated soils were used, in one experiment, the abundance of AMF was controlled by planting different plant genotypes, allowing an assessment of the effects in the context of a more realistic soil biological background as soil had not been sterilized.

In experiments using sterilized and re-inoculated soil to manipulate the presence of AMF, the abundance of soil biota other than AMF is strongly reduced. We added a microbial wash to re-introduce soil microorganisms to the sterilized soils (Koide and Li, 1989), but the microorganisms added are not likely to achieve abundances comparable to unsterilized field soil. Hence, the observed effects of AMF might be amplified compared to soil conditions where other soil biota are abundant.

By using different genotypes of tomato- a mutant showing reduced mycorrhizal colonization and a wildtype normally being colonized by AMF- we could avoid soil sterilization, as only the respective seed has to be planted to produce an AMF and a control treatment. Consequently, AMF find themselves in a more diverse soil ecological environment with soil microbes, as well as members of the soil mesofauna present (Rillig et al., 2008). With both approaches, we found AMF to reduce N_2O emissions. Hence, our data suggest that these findings might also be relevant in ecologically more realistic settings like in field situations. This has to be, however, specifically tested.

N_2O emissions are affected by a variety of interacting factors like soil N, C and O_2 availability and soil pH. Our data did not allow to detect a precise mechanism by which AM fungi reduce N_2O emissions. However, it suggests that a combination of effects that AMF exert on the plant soil system results in reduced N_2O emissions. Based on the results presented in this thesis and those published in the literature we present a conceptual model summarizing the potential ways by which AMF can contribute to reduced N_2O emissions (Fig. 3): these include reduced soil N availability (Chapter 1, Chapter 3, Bago et al. (1996); Johansen et al. (1993)), reduced soil water content causing increased oxygen availability (Chapter 1, Huang et al. (1985)), modifications in C cycling including reduced and

modified carbon allocation to rhizosphere soil, enhanced C input into bulk soil and enhanced CO_2 emissions (Chapter 1, Chapter 3, Marschner et al. (1997), Drigo et al. (2010); Hooker et al. (2007)), and a change in the community composition of denitrifying micro-organisms (Chapter 1, Amora-Lazcano et al. (1998); Veresoglou et al. (2012b)). Another factor reported to influence N_2O emissions, is soil pH. However, in our experiments, differences in soil pH were, even if partly significant, much smaller as pH differences reported to affect N_2O emissions in the literature.

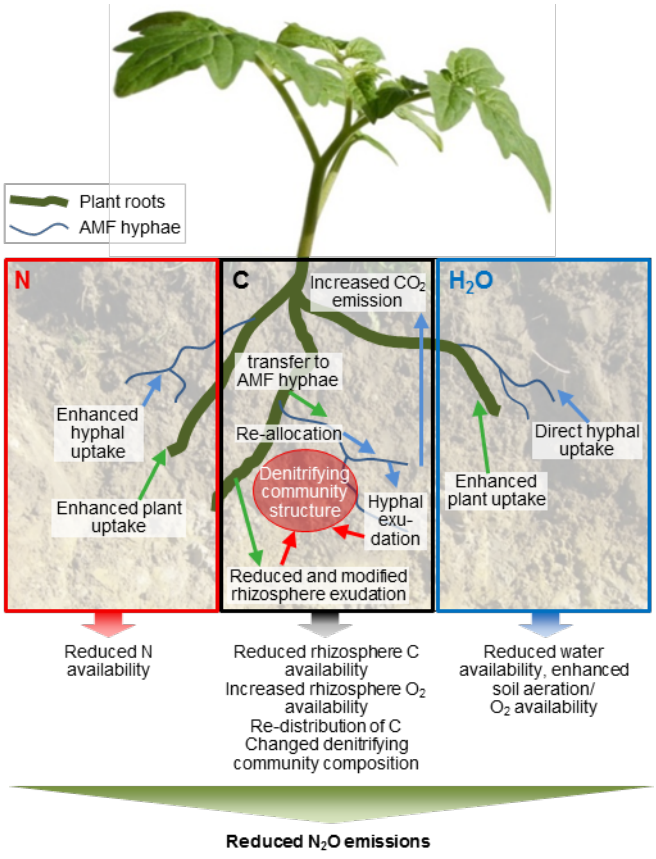

Figure 1: Conceptual model summarizing the most likely mechanisms how AMF reduce N2O emissions as suggested by our data and the literature (see text for details).

While N_2O is an intermediate, N_2 is the final product of denitrification. A reduction in denitrification related N_2O emissions implies either that total denitrification is lower with AMF , i.e. also the emissions of N2 are lower, or that the N_2O/N_2 ratio is reduced, implying that more N_2 is emitted. In Chapter 3, we attempted to quantify emissions of N_2. Due to technical issues, we were only able to quantify a certain fraction of N_2 emissions, namely N_2 molecules of mass 29 ($^{29}N_2$). Emissions of $^{29}N_2$ were significantly increased by AMF, suggesting that AMF increase denitrification efficiency, resulting in reduced emissions of N_2O and increased emissions of N_2. This also matches the observations made in chapter 1 that the abundance of genes involved in the emissions of N_2O were decreased, while the abundance of genes involved in N_2O reduction to N_2 were increased with increasing AMF abundance. However, our data do not allow to draw strong conclusions, as we lack data on total N_2 emissions.

Our results clearly show, that AMF reduce N_2O emissions from denitrification and suggest that AMF induce a higher denitrification efficiency resulting in reduced N_2O and enhanced N_2 emissions.

N_2O is a strong greenhouse gas and emissions of N_2 are the only pathway by which reactive N, that is excessively added to the biosphere through human activities, can be retransformed into unreactive, atmospheric N_2 (Schlesinger, 2009). These results are, thus, of potential importance for the mitigation of climate change and for the management of global N cycling.

(2) Can AMF reduce nutrient leaching from soil?

N leaching

Effects of AMF on the leaching of plant nutrients were investigated in Chapters 2,3 and 4. No consistent effects on N leaching was found across chapters. The biggest fraction of N leaching occurred in the form of NO_3^-. NO_3^- leaching was significantly reduced in chapter 3 and also in in the first year of chapter 4, but not in the second year. When we observed significant reductions in NO_3^- leaching, this was accompanied by strong increases in plant N nutrition suggesting that AMF effects on N leaching are predominately indirectly transmitted via increased plant N uptake.

Leaching of NH_4 was significantly reduced by AMF in chapter 2 and chapter 4, while in chapter 3 no effect of AMF on NH_4 leaching was observed. In chapters 2 and 4 sterilized soil was used, while in Chapter 3 we used non-sterile field soil. Other work reporting reduced NH_4 leaching by AMF was also performed with sterilized substrates (Asghari and Cavagnaro, 2011; van der Heijden, 2010), while one study also making use of a tomato mutant/wildtype pair and, hence, also using unsterile soil found no effect on NH_4 leaching(Asghari and Cavagnaro, 2012). As soil sterilization procedures have been shown to affect soil properties, including increases in NH4 availability (Endlweber and Scheu, 2006; McNamara et al., 2003), the difference in AMF effects on NH_4 leaching could be related to shifts in N dynamics induced by the sterilization procedure. Quantities of NH_4 leached were, however, very low compared to the total amounts of N leached. Taken together, these results imply that the extent to which AMF reduce the leaching of different forms of N is context dependent.

In chapters 3 and 4, apart from AMF, a variety of other guilds of soil biota was presumably present in the soil, because in Chapter 3 the soil was not sterilized and in Chapter 4 the soil biota inoculum contained AMF and other soil organisms. Under these conditions, we observed the biggest effects on plant growth, nutrition and on nutrient leaching by AMF. As indicated by a meta-analysis using data of more than 300 experiments, there seems to be a general trend towards bigger AMF effects on plant growth with increasing complexity of the soil biological background, e.g. with increasing abundance of non-AMF species present in the soil (Hoeksema et al., 2010). This might be related to interactive effects of AMF with non-AMF soil organisms. Interactions of members of the soil fauna with AMF were reported to result in positive effects on plant biomass (Gange, 2000; Harris and Boerner, 1990; Klironomos and Kendrick, 1995) and sometimes enhanced plant nutrition (Koller et al., 2013; Lussenhop, 1996). For example the results by (Koller et al., 2013) show, that interactive effects of Protozoa enhancing the mineralization of N from organic matter and of AMF effectively taking up the mineralized N and translocating it to the plants strongly enhanced plant N nutrition compared to the effects of AMF alone. Hence, the strong effects on N cycling in Chapter 3 and 4

could be related to synergistic interactions of AMF with soil biota involved in nutrient mineralization. Further targeted research is required to specifically test for these interactive effects.

In chapter 2, an inoculum-mixture of three known AMF species had been used for the experiment. In chapters 3 and 4, the AMF species present originated from the naturally occurring AMF communities of the soils used in the experiments and were not characterized further. It has been shown that, depending on the environmental conditions, species mixtures of AMF can positively affect plant production either through complementarity effects, (e.g. several species facilitating each other in their effects) or by selection effects (species mixture of higher diversity have a higher probability of containing a very effective species)(Wagg et al., 2011). Consequently, the differences observed in plant N uptake and N leaching losses between Chapter 2 and Chapters 3 and 4 could also result from different AMF species communities present in soil potentially resulting in stronger effects on plant N nutrition and N leaching. The conditions under which the strong potential of AMF to reduce N leaching can be exploited to enhance efficient nutrient cycling in agriculture have to be further investigated.

Although, the effect of AMF on N leaching was not very consistent, in chapters 3 and 4 it was quantitatively very significant, striking the big potential of AMF to reduce N leaching losses. Importantly, we found strong effects on N leaching in Chapter 4, where AMF effects in an agricultural crop rotation were investigated.

P leaching

Leaching losses of P were investigated in chapters 2 and 4. In chapter 2, we found reduced P leaching losses in presence of AMF from both soils tested. In the lysimeter-experiment in chapter 4, however, P leaching was increased by AMF. In the lysimeters, the soil column had a depth of 80 cm and consisted of a soil profile including subsoil. It has been shown, that P can be strongly sorbed in subsoils (Sinaj et al., 2002). P sorption dynamics in the subsoil can, hence, make the interpretation and comparability of P leaching results with the microcosm experiments in the greenhouse,

containing top soil only, difficult. P losses through leaching in greenhouse experiments, even if low already might be overrated compared to field situations where the soil columns are deeper and likely much P is sorbed in soil.

The increased P leaching the presence of soil biota and AMF in the Lysimeters of Chapter 4 might be related to members of the soil fauna and protozoa being present, likely increasing nutrient mineralization. The low, but significantly increased P leaching losses with soil biota could be considered as some "collateral damage" from enhanced nutrient mobilization leading to strongly enhanced plant P nutrition.

Leaching of non-mineral compounds

In Chapters 2 and 4, we observed AMF effects on leaching of organic N compounds and non-reactive P compounds. While other studies investigating effects of AMF on nutrient leaching had almost exclusively focused on leaching of dissolved mineral nutrient compounds, our results show for the first time that AMF possess the potential to reduce leaching of organic N and unreactive (i.e. not directly plant available) P compounds. Furthermore, we show that leaching of organic and unreactive nutrient compounds can be quantitatively important. For example in Chapter 2, we found fractions of organic N leaching up to 87% and fractions of unreactive P leaching up to more than 90% of total leaching losses.

(3) Can AMF contribute to sustainable agricultural practices by improving plant nutrition and reducing nutrient losses?

Effects on plant growth and nutrition

In the grass experiment of Chapter 1 and in Chapter 2, a grass species was used as host plant. Grasses are known to be rather unresponsive to AMF colonization. In Chapter 1, no differences in N content and biomass between the M and NM treatment were observed. In chapter 2, AMF slightly increased plant biomass and N and P contents in one soil type but not in the other. In the Tomato experiment

in Chapter 1, in Chapter 3, and also in the first year of Chapter 4 significant increases in plant biomass were observed. These differences could be related to plant identity. However, as already discussed for N leaching above, also the presence of soil organisms other than AMF in the soils of chapter 3 and 4, or differences in the AMF community composition could have resulted in the stronger effects on plant growth and nutrition in these chapters. In chapter 4, also the bigger soil volume could have played a role, also allowing deeper rooting of the plants. In greenhouse experiments using pots comprising a soil volume of 0.6l, Veiga et al. (2011) found no effects or even a reduction in maize biomass in pots inoculated with AMF compared to non-mycorrhizal controls suggesting that the growth response of plants may partly depend on soil volume available for root and AMF development.

Importantly, the biomass and nutrition of important crops was strongly increased in our experiments.

Ecological relevance of the findings

The four experiments conducted during this PhD thesis provide evidence that AMF can reduce emissions of N_2O, leaching losses of N and P, as well as strongly increase plant biomass and nutrition. We tried to increase the ecological relevance of the research conducted by mixing our experimental soils with less sand than commonly done in AMF research e.g. (Hodge et al., 2001; Jakobsen and Rosendahl, 1990; van der Heijden et al., 1998), by using microcosms comprising a relatively high soil volume and by adding a microbial wash to the experiments using sterilized soil to include natural microorganisms from the soils used and to balance out potential differences in the microbial communities from AMF- and non-mycorrhizal inoculums. Moreover, we additionally made use of a tomato mutant/wildtype pair to manipulate the presence of AMF, allowing the use of unsterilized soil and, consequently, the investigation of AMF effects with a diverse and abundant soil biological background present.

Furthermore, the Lysimeter experiment in Chapter 4 was an outdoor experiment in lysimeters comprising a soil volume of more than 200l and being filled with two soil horizons imitating the

natural soil profile. The experiment was exposed to natural climatic conditions. The soil biota inoculum used to inoculate the lysimeters comprised a natural AMF community and other soil organisms from the same soil used to fill the lysimeters. Hence, soil organisms found themselves under conditions resembling their natural habitat. However, despite the applied measures to increase ecological significance, our findings remain to be confirmed in field situations.

Field evidence

As a starting point, we present unpublished data from a long term field experiment investigating the effects of contrasting grassland management on N cycling (Flechard et al., 2005) to show that field situations exist, where reduced AMF abundance co-occurs with increased N_2O emissions. The intensive field was regularly fertilized with cattle slurry and mineral fertilizer, amounting to 200kg N $ha^{-1}yr^{-1}$, while the extensive field did not receive any external N inputs. Evaluation of the N_2O fluxes in 2010 revealed 12 fold higher N_2O emissions in the intensively managed field ($F_{1,6}$=9.45, P=0.022, Fig. 4a). Assessment of the root colonization with AM fungi showed that AM fungal abundance was decreased by 33% in the intensively managed field ($F_{1,6}$=7.62, P=0.033, Fig. 4b), in line with other studies showing reduced AM fungal abundance under intensive management (Helgason et al., 1998; Oehl et al., 2003a). These results confirm that reduced AM fungal abundance can occur coordinately with increased N_2O emissions in field situations. Previous studies have demonstrated that a reduction in AM fungal abundance lead to a decline of functions provided by AM fungi, such as the acquisition of nutrients (Johnson, 1993; van der Heijden, 2010). It is out of doubt that the strong differences in the fertilization regime mainly caused the strong differences in N_2O emissions. However, in addition to increased N availability, the detrimental effect of fertilization on AM fungi (Egerton-Warburton and Allen, 2000) might have contributed to increased N_2O emissions. Although not proving a causal relationship of AMF and N_2O emissions in the field, these results indicate, that field situations exist, where reduced AMF abundance co-occurs with increased N_2O emissions.

Further field based investigations are needed to confirm a causal link of the abundance of AMF with nutrient losses.

A correlative study investigating the role of soil biota in ecosystem processes on several agricultural sites across Europe (de Vries et al., 2013) recently found that the abundance of AMF correlated negatively with N leaching losses across all sites. Our results suggest that this correlation might be a causal relationship.

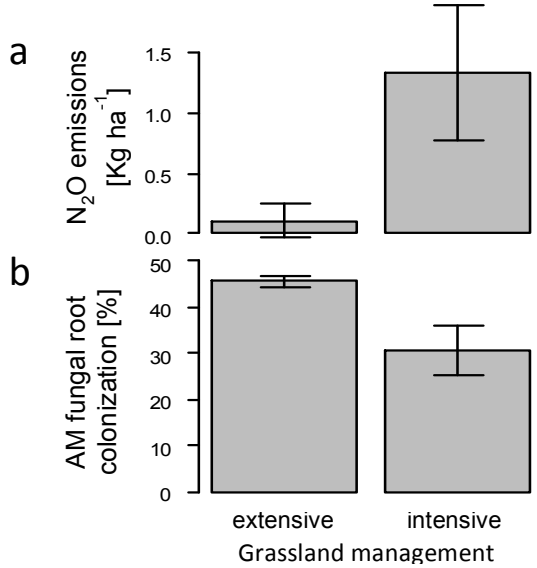

Figure 4: N_2O emissions (a) and AM fungal root colonization (b) under contrasting grassland management strategies measured in a long-term field experiment in Oensingen, Switzerland. Error bars represent ± 1 SEM (n=4).

To effectively use the potential of AMF in reducing nutrient losses and promoting plant growth with reduced resource inputs, management practices have to be applied that promote the abundance of AMF. The field data presented above and many reports in the literature show, that intensive agricultural management reduces the abundance and diversity of AMF communities (de Vries et al., 2013; Oehl et al., 2003a; Verbruggen et al., 2010). Consequently, less intensive management of agricultural fields would be one measure to enhance AMF abundance and to make use of their benefits.

In a Swiss long-term field trial comparing conventional and organic farming practices(Mader et al., 2002) it was found that nutrient input in the organically management fields was between 34 and 51% lower compared to the conventionally managed fields. Yields were, however, only reduced by 18% on average. Root length colonized by AMF was on average 40% higher in the organic farming systems. These results indicate a higher nutrient use efficiency in the organically managed systems that could well be explained by a higher abundance of AMF promoting efficient nutrient cycling and potentially also reducing nutrient losses. The major difference between the organic and conventional farming systems in this study were omission of chemical pesticides and application of a lower fertilization regime. It is well known that fertilizer applications negatively affect AMF communities and abundance (Egerton-Warburton and Allen, 2000; Kahiluoto et al., 2000) and that applications of fertilizers promote AMF species potentially providing less benefits for plant growth (Johnson, 1993).

In this field study, AMF were, however not specifically promoted. With targeted management practices to promote AMF communities in agricultural fields, it might be possible to obtain an even lower ratio of nutrient input to crop yield and, hence, stronger increases in resource efficiency. It is for example well known that farming practices like reduced or no-tillage favour AMF (Helgason et al., 1998) and lead to the development of AMF communities providing more beneficial ecosystem services (Köhl et al., 2014). Another measure to enhance AMF abundance is the introduction of AMF propagules with inoculants to agricultural fields. While this has been successfully tested in controlled field experiments (e.g.(Owusu-Bennoah and Mosse, 1979; Tawaraya et al., 2012), there remains many problems and questions that have to be solved before this approach could be used in large scale (Ijdo et al., 2011). Also breeding of host-plant/AMF combinations maximizing desirable functional traits leading to maximized resource efficiency would be an option to enhance the sustainability of cropping systems.

Conclusions

AMF could be key organisms to enhance the sustainability of agricultural systems by enhancing productivity and, as we showed here, reducing nutrient losses. Agriculture is globally one of the main source of excess nutrients entering the biosphere resulting in severe hazards on the earth system as a whole (Galloway et al., 2003; Rockstrom et al., 2009). By increasing the efficiency of nutrient cycling in the plant soil system, excess fertilizer application could be reduced being the main course for the strong nutrient inefficiency reported for agricultural systems (Liu et al., 2010; Smil, 1999). In this PhD thesis we showed, that increased nutrient use efficiency through AMF would not only benefit agricultural production but also the global environmental system. A reduction of nutrient leaching by AMF would reduce environmental hazards like water eutrophication. By reducing the emissions of the important greenhouse gas N_2O, an increase of AM fungal abundance could contribute to the mitigation of climate change as well as reducing the depletion of stratospheric ozone. If an increase in denitrification efficiency by AMF, resulting in enhance emissions of N_2, could be confirmed, this would open up new perspectives on the management of global N cycling. The promotion of complete denitrification to N_2 has been proposed as one of the main measures to reduce the negative impacts of the constant addition of reactive N to the biosphere through human activities on the earth system (Schlesinger, 2009).

AMF are a globally distributed group of soil microorgansims that form symbiotic relationships with the majority of land plants (Smith and Read, 2008) and that make up a substantial portion of microbial biomass in soil (Olsson et al., 1999). The results obtained in this PhD thesis imply, that the global AMF community possesses a tremendous importance for the functioning of the earth system. A small shift in the global abundance of AMF could have severe impacts on the emissions of greenhouse gases, the pollution of waterways and on plant diversity and productivity. AMF have to be seriously integrated into considerations on global sustainable development, as they could play a key role for the functioning of planet earth as we know it.

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Appendix

Soil biodiversity and soil community composition determine ecosystem multifunctionality

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Abstract

Biodiversity loss has become a global concern as evidence accumulates that it will negatively affect ecosystem services on which society depends. So far, most studies focused on the ecological consequences of aboveground biodiversity loss, yet a large part of Earth's biodiversity is literally hidden belowground. Whether reductions of biodiversity in soil communities belowground has consequences for the overall performance of an ecosystem remains unresolved. It is important to investigate this in view of recent observations that soil biodiversity is declining and that soil communities are changing upon land use intensification. We established soil communities differing in composition and diversity and tested their impact on eight ecosystem functions in model grassland communities. We show that soil biodiversity loss and simplification of soil community composition, impair multiple ecosystem functions including plant diversity, decomposition, nutrient retention and nutrient cycling. The average response of all measured ecosystem functions (ecosystem multifunctionality) exhibited a strong positive linear relationship to indicators of soil biodiversity, demonstrating that soil community composition is a key factor in regulating ecosystem functioning. Our results indicate that changes in soil communities and the loss of soil biodiversity threatens ecosystem multifunctionality and sustainability.

Significance Statement

Biological diversity is the foundation for the maintenance of ecosystems. Consequently it is thought that anthropogenic activities that reduce the diversity in ecosystems threatens ecosystem performance. A large proportion of the biodiversity within terrestrial ecosystems is hidden belowground in soils and the impact of altering its diversity and composition on the performance of ecosystems is still poorly understood. Using a novel experimental system to alter levels of soil biodiversity and community composition we found that reductions in the abundance and presence of soil organisms results in the decline of multiple ecosystem functions including plant diversity and nutrient cycling and retention. This suggests that belowground biodiversity is a key resource for maintaining the functioning of ecosystems.

Introduction

It has long been recognized that biodiversity can be the mechanism behind the performance of an ecosystem, particularly in communities of aboveground organisms (1-5). In soils belowground, however, the functioning of biodiversity is not well understood (6). Soils are highly diverse. It has been estimated that one gram of soil contains up to 1 billion bacteria cells consisting of tens of thousands of taxa, up to 200 m fungal hyphae, and a wide range of mites, nematodes, earthworms and arthropods (7, 8). This vast and hidden diversity contributes to the total terrestrial biomass and is intimately linked to aboveground biodiversity (9, 10).

In recent years several studies have shown that anthropogenic activities such as agricultural intensification and land use change, reduce microbial and faunal abundance and the overall diversity of soil organisms (11-13). This has triggered increasing concern that reduced biodiversity in soils may impair numerous ecosystem functions such as nutrient acquisition by plants and the cycling of resources between above and belowground communities (6, 11, 13, 14). However, so far research has largely focused on the effects of specific groups of organisms such as soil microbes (15, 16), mycorrhizal fungi (17, 18), and soil fauna (19, 20), or on large scale correlative analysis in the field

(13). However, soil organisms interact within complex food webs and therefore changes in diversity within one trophic group or functional guild may alter the abundance, diversity, and functioning of another (21, 22). Hence, it is important to know how changes in soil biodiversity and the simplification of the soil community composition influences ecosystem functioning. However, whether reductions of biodiversity in soil communities has consequences for the overall performance of an ecosystem remains unresolved. Moreover, recent studies show that aboveground plant diversity influences multiple ecosystem functions, defined as ecosystem multifunctionality (23). Yet, it is still unclear whether ecosystem multifunctionality is likewise influenced by soil biodiversity.

Here we manipulated soil biodiversity and soil community composition in model grassland microcosms simulating European grassland. We tested whether changes in soil biodiversity and soil community composition influenced multiple ecosystem functions. In order to manipulate soil biodiversity and soil community composition, we inoculated the grassland microcosms with different soil communities. The soil inoculum was prepared by fractionating soil communities based on size, using filters of decreasing mesh size (19). This method reduces the abundance of different groups of soil organisms at different mesh sizes, thus altering the community composition and the overall diversity of soil organisms simultaneously (19). In order to maintain the different soil community treatments and to prevent microbial contamination, we maintained the communities in self-contained microcosms in which we could restrict external contamination (24). We used the average standardized score of each soil community parameter as an index of soil biodiversity that incorporates the community compositional changes in concert with changes in soil biodiversity (see Materials and Methods). Here we refer to it as the soil biodiversity index, for simplicity. Additionally, the experiment was repeated for a longer time period to confirm initial results and include additional measures on ecosystem characteristics. We hypothesized that soil biodiversity loss reduces ecosystem functioning and multifunctionality. Specifically, we hypothesized that plant diversity, decomposition and the recycling of nutrients is impaired when the diversity and abundance of various groups of soil biota (e.g. fungi, mycorrhizal fungi, bacteria, nematodes) are reduced.

Results and Discussion

We successfully obtained a broad soil biodiversity gradient in our grassland microcosms (Fig. 1, SI Appendix Fig S1 – S2, Table S1). Some groups of soil organisms (e.g. nematodes and mycorrhizal fungi) were entirely eliminated within the gradient while fungal and bacterial communities showed reduced abundance and richness (Fig. 1). This resulted in an overall shift in soil community composition and in a decline in the diversity of soil biota in each soil community treatment along our gradient.

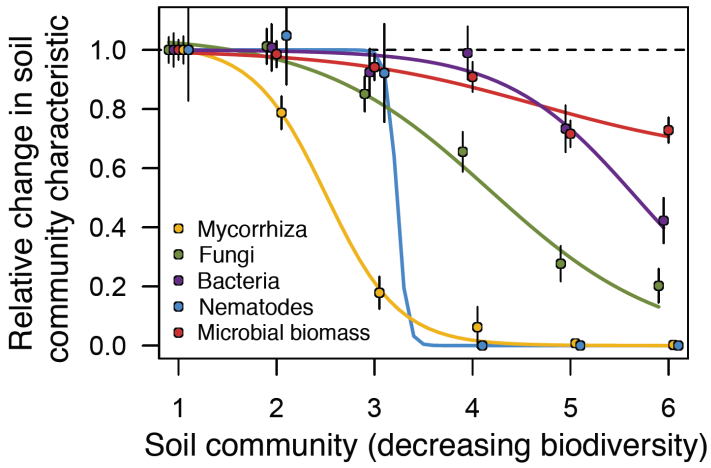

Fig. 1. Change in soil community characteristics in grassland communities with increasing simplification of soil communities based on size. Soil communities were established by filtering through different meshes: 1 ≤ 5000 μm, 2 ≤ 250 μm, 3 ≤ 50 μm, 4 ≤ 25 μm, 5 ≤ 10 μm, and 6 = sterilized soil. These measures reflect both abundance (nematodes, mycorrhizal colonization of plant roots, and microbial biomass) and richness (bacteria and fungal richness) of various guilds of soil organisms. Means ± SEM are expressed as a ratio of the most complete soil treatment (soil community 1 — dashed line), such that 0 represents no detection (see SI Appendix Fig. S1 for raw data). Where no error bars are shown for mycorrhiza and nematodes they were not detected in any replicate. Lines highlight the general trend in changes in the soil community characteristics along the gradient. Soil community characteristics measured in both experiments are pooled.

Changes in the soil communities across the gradient influenced various ecosystem functions (Fig 2). Among the ecosystem functions assessed, plant species diversity declined strongly with reductions in soil biodiversity and simplification of the soil communities (Fig. 2) supporting previous reports that plant community composition is driven by the diversity and species composition of

various groups of soil organisms (17, 19, 25). Legumes and forbs declined in productivity as soil biodiversity was depleted, while grasses increased in productivity in the most simplified soil communities, contributing up to 92 % of the net primary productivity (see SI Appendix Fig. S3 – S4). Carbon sequestration also declined along the gradient (Fig. 2). However, this effect was relatively small as this function is likely mediated more by a combination of plant and soil community characteristics than a direct function of soil biodiversity alone (26, 27).

The changes in soil biodiversity and soil community composition also influenced processes related to nutrient cycling. Changes in ecosystem processes that retain nutrients within the system are linked to the ability of soil organisms to breakdown organic matter and recycle liberated resources back into the aboveground community (10). Specifically, the decomposition of plant litter and the reincorporation of the nitrogen liberated from the litter back into aboveground plant tissues declined as overall soil biodiversity was reduced and with simplification of the soil communities (Fig. 2). Moreover, phosphorus loss through leaching after a simulated rain increased exponentially with successive simplification of soil communities reaching up to a 3-fold loss in the most simplified soil community (Fig. 2). These results support past observations and hypotheses suggesting that a greater diversity of soil organisms can enhance litter break down, reduce nutrient leaching losses and maintain resource turnover between above and belowground communities (10, 11, 14). The loss of nitrogen via N_2O emissions also increased up to 6 fold in the second most simplified soil community (Fig. 2). This demonstrates that the simplification of soil biotic communities alters nitrogen transformation processes in the soil, resulting in increased emission of N_2O, which is an important greenhouse gas (28).

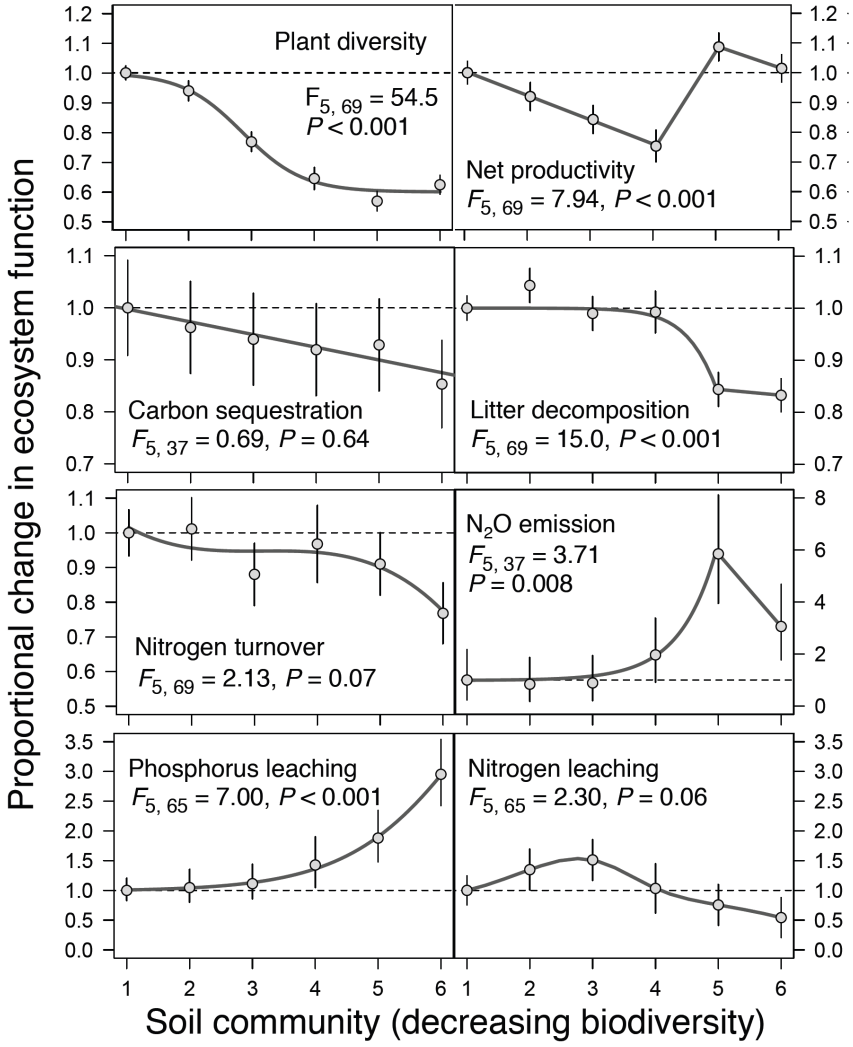

Fig. 2. Change in ecosystem functions in grassland communities along the continuum of increasingly simplified soil biotic communities. Means ± SEM of plant productivity (g), plant diversity (Shannon index), N turnover (shoot $\partial^{15}N$), Decomposition (%), C sequestration (soil $\partial^{13}C$), N leaching (mg), P leaching (mg), N_2O emissions (mg m^{-2}) are expressed as a ratio of the most complete soil treatment (soil community 1 dashed line) such that values below 1 represent a reduction and values above 1 indicate an increase in the ecosystem function (see fig. S4 for raw data). Lines highlight trends in the changes in ecosystem functions across the gradient. Ecosystem functions measured in both experiments are pooled (see SI Appendix Fig. S6 - S7, and Table S1 for the results of the individual experiments). Soil communities are based on organism size as described in Fig. 1.

In order to assess the overall performance of the grassland microcosms we averaged the standardized scores (z-scores) of all ecosystem functions (shown in Fig. 2) to obtain a single index of

ecosystem multifunctionality (23). We combined the soil community characteristics (shown in Fig. 1) in the same manner to obtain a single index reflecting the soil biodiversity within the microcosms created by filtering. Overall, the changes in ecosystem multifunctionality showed a strong positive relationship to the average of our indicators of soil biodiversity (Fig. 3), indicating that changes in soil biodiversity impact ecosystem multifunctionality. The substantial proportion of variation in ecosystem multifunctionality explained by the soil biodiversity index indicates that the soil community characteristics measured were appropriate indicators of soil biodiversity in our system. Importantly, ecosystem multifunctionality, as well as the index of soil biodiversity, did not vary strongly between the two initial levels of soil community simplification (e.g. soil communities consisting of organisms up to 5000 μm or 250 μm) and a strong reduction in ecosystem multifunctionality was only observed in highly simplified soil communities (SI Appendix Fig. S7). This supports the theory that at higher levels of soil biodiversity, ecosystem functions are robust to changes in soil biodiversity and composition of soil biota (21). Moreover, as soil communities became increasingly simplified, the loss or strong suppression of key groups of soil organisms (e.g. mycorrhizal fungi and nematodes) corresponded with an abrupt shift in many of the ecosystem functions (see Fig. 2 and SI Appendix Fig. S8). This highlights that broad-scale changes in the soil community can be tightly linked to the overall functioning of the ecosystem and that ecosystem functioning is more sensitive to changes in the presence and abundance of various soil organisms when overall biodiversity is low (21).

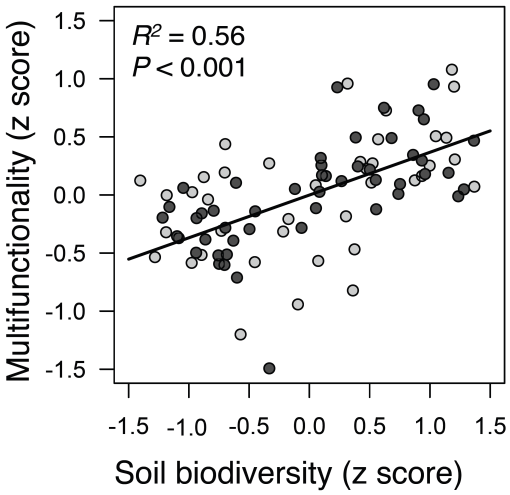

Fig. 3. Ecosystem multifunctionality index in relation to the soil biodiversity index. Lightly shaded points represent grassland communities in experiment 1 and darkly shaded points indicate grassland communities in experiment 2. The overall regression is shown pooled for both trials since there was no difference between trials in the overall relationship between the soil biodiversity (the combined measures shown in Fig. 1) and ecosystem multifunctionality (the combined ecosystem functions shown in Fig. 2). The relationship of individual ecosystem functions to the soil biodiversity score is shown in SI Appendix Fig. S5 and changes in the two indices across the gradient of soil communities are shown in SI Appendix Fig. S6.

Our results were obtained in two independent experiments and results of both experiments were similar, pointing to the robustness of our findings. The effects of changes in soil biodiversity and community composition on decomposition of plant litter and nutrient turnover were stronger in the second experiment (SI Appendix Fig. S8), which was the longer-lasting experiment. This suggests that the consequences of simplified soil community composition and reduced soil diversity may become progressively more inhibiting as time passes. Additionally, since plant diversity is also a driver of ecosystem multifunctionality (3-5, 23), the strong effects of soil organisms on plant diversity, observed here and elsewhere (15-20), could indirectly influence a number of other ecosystem functions such as nutrient availability (23) and C sequestration (26). A path analysis indeed indicated that effects of soil biodiversity and composition on measures of nutrient leaching were, in part, indirect and mediated by soil biodiversity induced changes in plant diversity and productivity (SI Appendix Fig S9 – S10 and Table S2-S3). Further path analyses assessing the direct and indirect associations between the individual soil community characteristics and ecosystem functions indicate

that different components of the soil community differentially influence the various ecosystem functions (SI Appendix Fig. S4 – S5, Tables S4 – S5). The differences in association between groups of organisms with different ecosystem functions suggests that the changes in diversity and abundance, both within and between groups of soil organisms, is likely an underlying mechanism behind the improved ecosystem multifunctionality with greater soil biodiversity (21, 22).

Two decades of biodiversity research have shown that aboveground plant diversity is a key driver of ecosystem functioning in a wide range of ecosystems (2-5). Our research extends this observation to the belowground environment, showing that a reduction of soil biodiversity and changes in soil community composition impacts, not only on the associated plant community, but also on a number of key ecosystem processes that are necessary to maintain overall ecosystem performance. These findings are in line with a recent large-scale correlative field study that indicates that soil food web properties are associated with ecosystem services across various European land use systems (13). The predicted suppression of soil biodiversity due to chronic disruptions to soil communities through intensified anthropogenic activities (11-13) coupled with climate change are likely to negatively influence the performance of multiple ecosystem process (6). Thus, the protection of soil biodiversity is a key issue to be considered in further detail for the sustainability of terrestrial ecosystems.

Materials and Methods

Microcosms, Substrate, Soil and Plant Communities. Experimental grassland microcosms were established under sterile conditions in closed growth chambers. Incoming air and water entered the microcosms though purifying filters to prevent outside contamination (24; see SI Appendix). Microcosms measured 23.5 cm in diameter and had a rooting depth of 12 cm. Each microcosm was filled with 6 kg of a standard sterile soil (96% soil volume) and an inoculated soil community (4% soil volume). Different soil community treatments were prepared by sequentially sieving 250 g field soil

through a series of decreasing mesh sizes: soil organisms ≤5000 µm, ≤ 250 µm, ≤ 50 µm, ≤ 10 µm, and sterile soil.in experiment 1. In experiment 2 an additional < 25 µm soil community treatment was added to the design. Each soil community treatment was replicated 8 times in both experiments with the exception of 10 replicates of the sterilized soil community in experiment 2, bringing the total experimental units in the two experiments to 40 and 50 respectively. The reduction of soil communities by filtering removes guilds of soil organisms as well as dilutes out their abundance with each additional filter (19, 29).

In each microcosms a plant community consisting of 40 individual plants comprising 10 species that are typical of temperate European grasslands (30) were planted: legumes (5 individuals of *Trifolium pratense* and 5 of *Lotus corniculatus*), grasses (4 *Lolium multiflorum*, 5 *Poa annua* and 5 *Festuca pratensis*) and forbs (3 *Prunella vulgaris*, 2 *Senecio jacobea*, 4 *Plantago lanceolata*, 3 *Achillea milleflorum*, and 4 *Capsella bursa-pastovis*). Microcosms were maintained in the greenhouse. The experiment was carried out twice; for a growth period of 14 weeks (experiment 1) and for a growth period of 24 weeks (experiment 2).

Soil Community Characterization. At the end of each experiment, and after 12 weeks for the second trial, soil was removed for molecular and microscopy analyses (see SI Appendix for details). Bacterial and fungal community composition was determined using ribosomal internal spacer analysis (RISA) (31-33). Roots were collected and scored for the absence/presence of arbuscular mycorrhizal colonization using an intersect-transect method for 100 intersections (34). The number of nematodes was assessed in a 100g soil sample (35) and Soil DNA was used as an indicator of soil microbial biomass as it corresponds well with other methods that reflect microbial biomass (36, 37).

Ecosystem Functions. At the final harvest, plant shoots were cut at the soil surface, the number of individuals of each species harvested was counted, and shoot biomass was determined. At 12 weeks in the second experimental trial, plants were harvested at 5 cm above the soil surface to simulate

hay making; typical for many European grasslands, and the number of plants per species harvest was recorded. Net plant productivity was measured as the total plant aboveground biomass. The Shannon-Wiener index of diversity was calculated using the aboveground biomass per individual plant harvested of each species as the surrogate for abundance in the equation.

Litter decomposition was assessed with litterbags containing ^{15}N labeled sterilized *Lolium multiflorum* shoots that were added to microcosms at the start of the experiment. The ability for a plant to acquire N through the mineralization of its litter is defined here as "N turnover" and was estimated using the $\delta^{15}N$ signal in the *L. multiflorum* shoots at the end of the experiment. At the final harvest, microcosms were watered to saturate the soil to roughly 10 % beyond the water holding capacity of the soil to induce leaching. Leachate percolating through the soil column was collected from a small outlet at the bottom of the microcosm and was assessed for nutrient concentrations (PO_4, total P, NO_3, NH_4) as described elsewhere (38, 39). Fertilization and water saturation of soil post rainfall events not only facilitate nutrient leaching, but can also initiate the denitrification related production of N_2O, an important greenhouse gas (40). Hence, N_2O production was measured at the end of the second experiment after fertilizer addition and soil saturation with water. At 48 and 24 hours prior to the final harvest of microcosms in the second experiment 40 mL of $^{13}CO_2$ (99%^{13}C) gas was injected into each microcosm and below ground ^{13}C allocation was measured. See SI Appendix for further details on all measurement procedures.

Soil Biodiversity and Multifunctionality Indices. All soil community and ecosystem function data from each harvest period were standardized by z transformation (overall mean of 0 and standard deviation of 1) and used in all subsequent calculations and analyses. This removed overall differences between trials and harvest time points and simultaneously equalized the variance among measures and sampling time points. Subsequently, the average of all standardized ecosystem functions (shown in SI Appendix Fig. S6) was used as an index of ecosystem multifunctionality following the approach used by Maestre et al. (23). Data for ecosystem functions where greater values reflect a more

undesirable aspect of the ecosystem (increasing nutrient leaching and N_2O production) were multiplied by –1 (inverted around the 0 mean) to maintain directional change with other ecosystem functions such that a decline from their desirable state corresponds to increasingly negative values. By doing this, the general difference among soil community treatments in overall ecosystem functioning could be more easily assessed.

A soil biodiversity index was calculated from the average of all standardized soil community characteristics and used as a general indicator of soil biodiversity and compositional changes. This soil biodiversity index includes measures of richness (bacterial richness, fungal richness) and the relative abundance of guilds of soil organisms (number of nematodes, root colonization by arbuscular mycorrhizal fungi, and an estimate of soil microbial biomass). Thus the biodiversity index calculated here parallels typical biodiversity indicators by combining measures of richness and relative abundance (41). Note however, that more ecosystem functions (carbon sequestration and N_2O emissions) and soil community characteristics (nematode abundance) were measured in the second experiment and are incorporated into the biodiversity and multifunctionality indices of the second experiment even though absent from the first.

Data analyses. All data on ecosystem functions and soil community characteristics were assessed for variation among soil community treatments in a mixed effects model using pooled data from both experiments as well as separately for each experimental trial to determine overall effects as well as differences between trials. The replicate block by which each microcosm was harvested, was used as the random effect. Regressions were also performed using mixed effect models to test whether ecosystem multifunctionality and individual ecosystem functions could be explained by the soil biodiversity index as well as whether this relationship depended upon the experimental trial (SI Appendix Fig. S6).

In addition to the method of averaging z scores of soil community characteristics, we used partial least squares path modeling to infer potential direct and indirect effects of soil biodiversity on

various ecosystem functions (42). In the path models soil biodiversity was constructed as a latent variable using the measured soil community characteristics as reflective indicators of soil biodiversity. Since all measures of soil biodiversity and ecosystem functions were strongly influenced by the different soil community treatments we assessed the variation in the measures of soil biodiversity as a direct effect of the variation in all ecosystem functions in the path model. However, the loss of soil biodiversity may have indirectly resulted in changes in some ecosystem functions. Specifically, the effect of the different soil communities on plant productivity and diversity may have consequently influenced carbon sequestration and nutrient losses through leaching (43, 44). Additionally we also assessed the effects of litter decomposition and N turnover on nutrient losses from the system (see SI Appendix for details on all models presented in Fig. S9 – S12 and Tables S2 – S5).

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Supporting Information

Materials and Methods

Microcosms, Substrate, and Soil Communities

Grassland microcosms were established under sterile conditions in closed growth chambers (24). To avoid any outside greenhouse-borne microbial contamination, incoming air was filtered through a hydrophobic filter with a pore size of 0.2 µm (Millex®-FG_{50}; Millipore Corporation, Billerica, USA) and water was filtered through a hydrophilic filter with a 0.22 µm pore size (Millex®-GP_{50}; Millipore Corporation, Billerica, USA). Microcosms were assembled, inoculated, and planted within a laminar flow hood. All parts used for the microcosms were sterilized by autoclaving for 30 min at 121 °C, with the exception of the Plexiglas tops and the PVC microcosm bottoms. The bottom and top of the microcosms were sterilized by submersing in 0.5% sodium hypochlorite for 20–30 minutes, then in 70% Ethanol with a few drops of Tween 20 for a few minutes and air-dried within the Laminar flow hood.

Each microcosm had a 1-cm deep layer of 1 cm diameter sterilized (90 min at 121 °C) quartz stones at the bottom with a 0.5 mm propyltex mesh (Sefar AG, Heiden, Switzerland) to aid in the collection of leachate (see below). Each microcosm was filled with 6 kg (dry mass) of a 1:1 quartz sand:field soil mix that was sterilized by autoclaving for 90 min at 121 °C. The field soil used was collected from a grassland located at the Agroscope Reckenholz research station in Zürich, Switzerland (47° 25’ 38.71’’ N, 8° 31’ 3.91’’ E) and was sieved through a 5 mm mesh before mixing. This same field soil was used for creating the gradient of soil communities via sieving out soil communities based on size. In experiment 1 there were five soil community treatments and in experiment 2 there were six, each being replicated 8 times with the exception of the sterile (0 µm) soil community in experiment 2 which had 10 replicates for a total of 40 and 50 experimental units respectively (also see Materials and Methods in the main text). The soil substrate in the microcosms, measured post inoculation, had a pH of 7.56 (SEM = 0.01) with 9.54 (SEM = 0.79) $mg \cdot kg^{-1}$ of inorganic N (NO_3^- and NH_4^+) determined by a Skalar segment flow analyser (Skalar, Breda, NL) after extraction

with 0.0125M $CaCl_2$. Plant available P_2O_5 and K_2O, extracted by CO_2 -saturated water, was 1.25 (SEM = 0.01) $mg \cdot kg^{-1}$ and 0.61 (SEM = 0.003) $mg \cdot kg^{-1}$ respectively. The ammonium acetate-EDTA (pH 4.65) extracted amounts of Ca, P, K and Mg in $mg \cdot kg^{-1}$ were 6.51 x 10^3 (SEM = 0.04 x10^3), 21.8 (SEM= 0.32), 15.7 (SEM = 0.11), and 4.88 (SEM = 0.03), respectively. In all cases no soil characteristic listed above varied greatly among treatments (all $F_{4,5} < 2.05$ and $P > 0.20$).

Plant Community and Growing Conditions. Seeds of each plant species (*Trifolium pratense, Lotus corniculatus*, *Lolium multiflorum*, *Poa annua*, *Festuca pratensis*, *Prunella vulgaris*, *Senecio jacobea*, *Plantago lanceolata*, *Achillea millefloru*m, and *Capsella bursa-pastovis*). were surface sterilized by agitation in 50 ml of 2.5 % Sodium hypochlorite and Tween 20, for 10 min followed by rinsing with sterilized dH_2O. Seeds were germinated 1% water-agar media in Petri dishes. Since germination times varied among species, seed preparation was staggered so that germination dates of all species coincided. Within 48 hours post germination seedlings with no visible signs of microbial contamination were transplanted into 40 evenly spaced positions, at random, in each microcosm in a laminar flow chamber to avoid microbial contamination. Due to the time required to setup and collect data during harvests, the setup and harvesting of microcosms was performed in replicate blocks, such that each block of microcosms was harvested and setup on the same day. Microcosms were maintained in the greenhouse and under natural light subsidized by 400-W high-pressure sodium lamps to maintain a light level above 300 W/m^2 during the 16 h / 25 – 30 °C days. Microcosms were watered with dH_2O every 48–72 hours to maintain soil moisture in the range of 10–20 % by weight (65–85 % water holding capacity of the soil).

Soil Community Characterization. At the end of each experiment, and after 12 weeks for the second trial, soil was mixed carefully and a 1 kg soil sample was removed for molecular and microscopy analyses. At each sampling time point a 500 mg subsample of each soil sample was then used for DNA extraction using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Switzerland) following the

manufactures instructions. Soil extracted DNA was quantified using the Quant-iT™ PicoGreen® (Molecular Probes, Eugene, OR) on a Cary Eclipse Fluorescence Spectrophotometer. Soil DNA was used as an indicator of soil microbial biomass since it corresponds well with other methods that reflect microbial biomass (36, 37).

Extracted soil DNA was diluted to 10 ng / µl and used as template in PCRs. Bacterial community profiles were generated using primers bRISArev and bRISAfor (FAM-labelled) with the cycling conditions and reagent concentrations outlined elsewhere (31). For fungal community profiles the primers fRISArev and fRISAfor (FAM-labelled) were used following the reagent concentration and cycling conditions outlined elsewhere (32). Two µl of the PCR products were mixed with 12 µl HiDi-Formamid and 0.2 µl MapMarker® 1000 (BioVentures, Murfreesboro, TN) as the size standard and subject to fragment analysis using an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Run conditions were set to injection time of 30 s at 1.5 kV and 10 s with a run time of 3000 s at 10 kV. Unambiguous peaks of amplified DNA fragments with a minimum threshold intensity value of 20 florescent units were characterized based on their relative migration units using GenMarker 1.5 genotyping software (SoftGenetics LLC, State College, PA) and defined as operational taxonomic units (OTUs). Bacterial and fungal richness was assessed as the number of OTUs (scorable peaks) per microscosm.

At the final harvest in both experiments, roots were washed free of soil, cut into small pieces, and root fragments were randomly collected to obtain roughly 3 g of fresh root. The sampled roots were fixed in 50 % ethanol, cleared with 10 % KOH in a water bath of 70 °C for 40 minutes, and then stained with a 5 % pen ink – vinegar (5 % acetic acid) solution (45) for 20 minutes. Processed roots were scored for the absence/presence of arbuscular mycorrhizal colonization using an intersect-transect method for 100 intersections (34).

Nematodes were extracted an numerated from a 100 g subsample of fresh soil collected from at the end of the second experiment using a suspension – centrifugation method (35). This was done by homogenizing the soil in a sucrose solution with a specific gravity of 1.18, centerfuging

inorganic debris to the bottom while suspending organic material, including organisms, toward the surface. The supernatant was then removed and diluted with water to reduce the specific gravity and precipite soil organisms with additional centerfugation. Nematodes in percipate were numerated and the number of nematodes per g of fresh soil was calculated. It was noted that this method also extracted other soil invertebrate fauna. However, these were only observed in a few microcosoms of the 5000 μm and 250 μm communities and were mainly Rotifera.

Ecosystem Functions. Litter decomposition was assessed using two 6 cm x 6 cm litterbags made of propyltex mesh (mesh size = 0.5 mm) that were inserted into each microcosm just below the soil surface. In experiment 1, each litterbag was filled with 18 mg sterilized *Lolium multiflorum* shoots enriched with the nitrogen isotope ^{15}N ($\delta^{15}N=17.2 \times 10^2$ ‰). To improve ^{15}N detection 1.0 g of sterilized ^{15}N enriched ($\delta^{15}N=9.41 \times 10^2$ ‰) *L. multiflorum* shoots were added to each litterbag. The mass of the remaining litter within the bags was determined at the end of each experiment and litter decomposition was calculated as the remaining mass expressed as a percentage of the original litter mass. Nitrogen release from the *L. multiflorum* leaf litter and subsequent reabsorption by *L. multiflorum* plants and incorporation back into aboveground biomass was determined by measuring $\delta^{15}N$ values in *L. multiflorum* shoots at each harvest. This ability for a plant to acquire N through the mineralization of its litter, defined here as "N turnover", was estimated using the $\delta^{15}N$ signal in the *L. multiflorum* shoots at the end of the experiment. We used *L. multiflorum* as a bio-indicator for this process of cycling organically derived N, since it was dominant in all microcosms (see SI Appendix Fig. S4) and is a strong competitor for plant available forms of nitrogen.

At the final harvest microcosms were watered to saturate the soil to roughly 10 % beyond its water holding capacity to induce leaching. Leachate percolating through the soil column was collected from a small outlet at the bottom of the microcosm and was assessed for nutrient concentrations (PO_4, NO_3, NH_4) as described elsewhere (38,39). Total P in the leachate was determined following the molybdate blue method (39) after Oxisolv® (Merck, Darmstadt, Germany)

oxidation using a spectrophotometer (Helios Gamma, Thermo Scientific, Digitana AG, Switzerland). The organic P leached was calculated as the difference between total and PO_4-P (inorganic P) leached. Leachate samples from one of the 8 replicate blocks in experiment 1 were compromised during collection and 5 samples are therefore missing from the data set.

Leachate concentrations of NO_3 and PO_4 in experiment 1 were frequently below detection level (see SI Appendix Fig. S4), causing large variation among replicates. Hence, in the second experiment, a 50 ml nutrient solution was given to the microcosms three days before watering in order to improve the ability to detect nutrients leached as well as to aid in the measurement of N_2O emissions (see below). The nutrient solution corresponded to a fertilizer application of 60 kg N per ha and 10 kg P per ha and contained 381 mM KNO_3, 29 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.4 mM $CaCl_2$, 25 µM KCl, 12.5 µM H_3BO_3, 1 µM $MnSO_4$, 1 µM $ZnSO_4$, 0.25 µM $CuSO_4$, 0.25 µM Na_2MoO_4 and 10 µM Fe-(Na) EDTA.

Fertilization and water saturation of soil post rainfall events not only facilitate nutrient leaching, but also initiate the production of N_2O, an important greenhouse gas (40). Hence, N_2O production was measured at the end of the second experiment after fertilizer addition and soil saturation with water. N_2O fluxes were measured by cycling microcosm air through a TEI 46c automated N_2O analyser (Thermo Fisher Scientific, Waltham, US) for a period of 10 minutes 3 times per day over 3 days starting immediately after the simulated rain prior to harvest at 24 weeks. The N_2O fluxes were integrated over this period by linear interpolation between single measurements and the total N_2O emitted was used as ecosystem function since N_2O represents nutrient loss as well as an important greenhouse gas (40)**.**

At 48 and 24 hours prior to the final harvest of microcosms in the second experiment 40 mL of $^{13}CO_2$ (99%^{13}C) gas was injected into each microcosm through the purifying air intake filter and the air exhaust and intakes were sealed off for a 5-hour period. This enabled plants to acquire $^{13}CO_2$. In order to assess ^{13}C allocated below ground, four soil cores, 2.5 cm in diameter, were taken to the depth of the substrate and included fine plant roots. These were mixed carefully, lyophilized and

milled into a fine powder. Inorganic carbon was removed prior to stable isotope analysis by acid fumigation (46). Moistened subsamples were exposed to the exhalation of HCl in a desiccator overnight and dried at 40°C before analysis. Samples were analysed by isotope ratio mass spectrometry (Thermo Finnigan Delta plus XP coupled with a Flash EA 1112 Series elemental analyzer; both instruments supplied by Thermo-Finnigan, Waltham, MA, USA) at the university of Basel, Switzerland. The $\delta^{13}C$ in parts per thousand relative to internal standards V-PDB was calculated as $(R_{sample} / R_{standard} - 1) \times 1{,}000$, where R is the ratio of ^{13}C to ^{12}C. This was then used as the indicator of the carbon sequestration ability of each microcosm.

Data analyses. Due to the time required to setup and collect data during harvests, the setup and harvesting of microcosms was performed in replicate blocks, such that each block of microcosms was harvested and setup on the same day. All data transformations and statistics were done using R software (version 2.13.0; The R Foundation for Statistical Computing 2011).

All data on ecosystem functions and soil community characteristics were first assessed separately for each experimental trial for variation among soil community treatments in a mixed effects model with the replicate block by which each microcosm was harvest as the random effect. Secondly, each soil community and ecosystem function were assessed for whether the variation in their response to the soil community treatments was dependent on the experimental trial by using the pooled data from both experiments (using the standardized z scores). This was done using data from the five soil community treatments that were common in both trials (soil community filtering treatments < 5000 µm, < 250 µm, < 50 µm, < 10 µm, and sterile). Finally, the pooled data were assessed in mixed effects models with the experiment and the block within each experiment as random effects. Ecosystem functions and soil community characteristics were assessed for differences among soil community treatments using pooled data from both experiments. Regressions were also performed using mixed effect models to test whether ecosystem multifunctionality and individual ecosystem functions could be explained by the soil biodiversity index as well as whether

this relationship depended upon the experimental trial (SI Fig. S6). Plant mortality was added as a covariate to the models assessing plant diversity and net primary productivity to control for plant density dependence in these measures. Log transformations were used to improve the homoscedasticity in all nutrient-leaching and N_2O-emission data.

In order to visualize changes in the soil community and ecosystem functions across the filtered soil community treatments (presented in Fig. 1 and Fig. 2 and in SI Fig. S8), the estimated model means and s.e. limits above and below the estimates were back-transformed to their original scale and expressed as a ratio of the most complete soil community treatment (5000 μm). This allows for ease of interpretation of the changes in the measured ecosystem data in proportion to the performance of ecosystem characteristics when soil communities are not sieved (soil organisms < 5 mm in size).

PLS Path modeling was performed in R using the package "plspm", see (42), to infer possible indirect versus direct effects of soil biodiversity on ecosystem functions. All models were constructed using the z-score standardized data and log transformed nutrient leaching and N_2O emission data as described above. The data from both experiments was pooled using only the variables that were common to both experiments. A second path model was constructed using only the data from experiment 2 as additional variables were measured in that experiment (e.g. nematodes) that could not be included when pooling the data of the two experiments. Furthermore it was the more comprehensive and longer lasting of the two experiments. For both models soil biodiversity was constructed as a latent variable using the measured soil community characteristics as reflective indicators; hence the soil community characteristics "reflect" the abstract measure of soil biodiversity. Path coefficients for models were assessed for a difference from 0 by bootstrapping using 1000 resamples to assess the precision of each path, as well as the total effects of soil biodiversity (combined direct and indirect effects) on each ecosystem function.

Models were constructed with all direct paths from the soil biodiversity latent variable to each individual ecosystem function. Indirect effects of soil biodiversity through plant diversity and

productivity on all nutrient leaching were also included following the hypothesis that plant diversity and community productivity would reduce nutrient leaching based on past studies (43, 44). Additionally, it was hypothesized that greater decomposition of organic matter by soil biota could increase nutrient losses through leaching (47), while greater N turnover through direct N uptake by plants may be the mechanisms by which nutrients are kept within the system (48). Therefore these paths were also assessed. Finally, nutrient turnover in our study is a measure of the ^{15}N found in *L. multiflorum* leaves that originated from previously labeled *L. multiflorum* leaf litter that was also used to measure the decomposition of organic biomass. Therefore we assessed the indirect effect of soil biodiversity on N turnover through the decomposition of the leaf litter relative to a direct effect of soil biodiversity following the hypothesis that greater decomposition leads to greater cycling of N back aboveground (N turnover). Models were then simplified by excluding the paths among ecosystem functions that did not fit the direction of the hypotheses (e.g. greater decomposition should not lead to less N turnover). Secondly, path coefficients that were not significantly different, or marginally significant, from 0 were removed in order to achieve the most parsimonious model for our system.

We also used a PLS path analysis in order to provide some inference as to the relative direct and indirect effects of the individual soil community characteristics. The same path modeling procedure was used as mentioned above. However, it should be noted that the effects between ecosystem functions and individual soil community are only associative as certain measures may reflect changes in other trophic groups not measured and the experiment was not designed to address specific hypothesis between the individual soil community characteristic and a given ecosystem function.

Additional References SI Materials and Methods

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SI: Figures

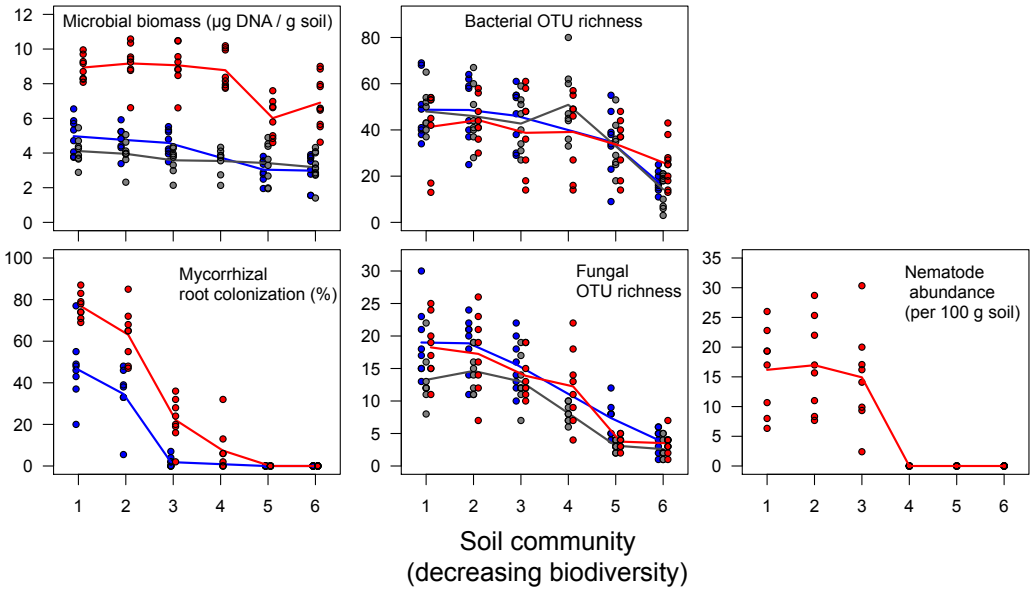


Fig S1. Data for the different in soil community characteristics measured in grassland microcosms with six different soil communities. The data for experiment 1 (blue lines and dots), experiment 2 after 12 weeks (grey lines and dots), and experiment 2 after 24 weeks (red lines and dots) are shown. Lines show the trend between consecutive means for each filter treatment. Points are staggered for clarity. Soil communities were established by filtering through different meshes: 1 ≤ 5000 µm, 2 ≤ 250 µm, 3 ≤ 50 µm, 4 ≤ 25 µm, 5 ≤ 10 µm, and 6 = sterilized soil. Note, for experiment 1 no data are shown for treatment 4 (soil communities ≤ 25 µm) because this treatment was not included this experiment. The average response of both experiments is shown in Fig. 1 in the main text.

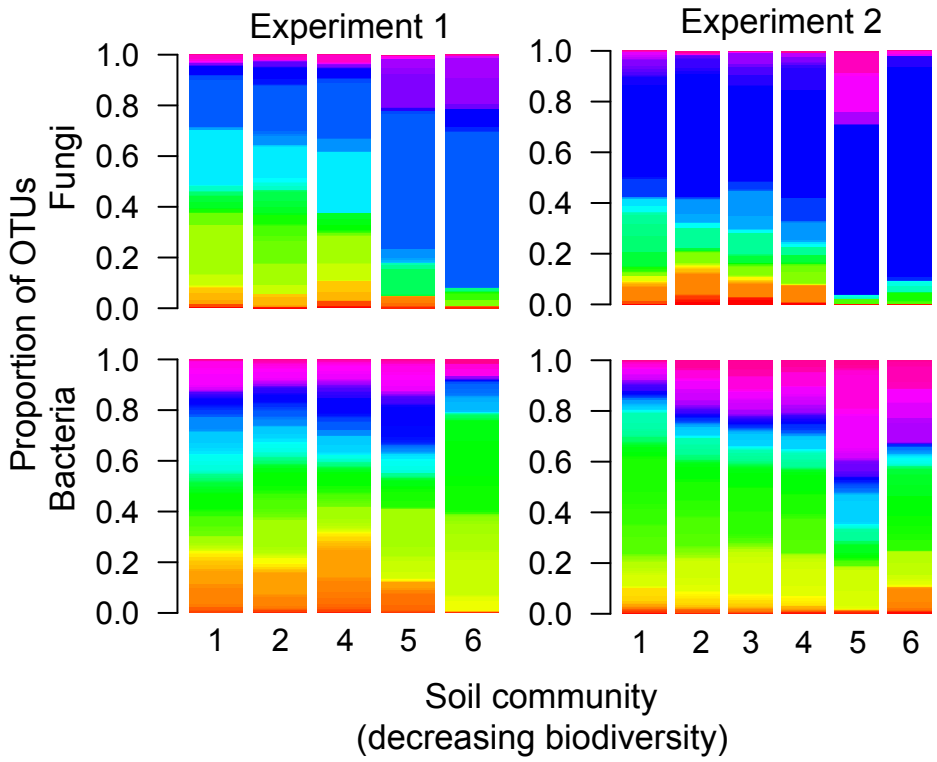


Fig. S2. The average proportion of individual fungal (top panels) and bacterial (bottom panels) OTUs that were detected at the end of each experiment are shown for each soil community treatment (1 ≤ 5000 μm, 2 ≤ 250 μm, 3 ≤ 50 μm, 4 ≤ 25 μm, 5 ≤ 10 μm, and 6 ≤ sterilized soil). Different OTUs are indicated by different colors for the visualization of the changes in the community composition and evenness. Note that similar colors between panels do not indicate the same OTU.

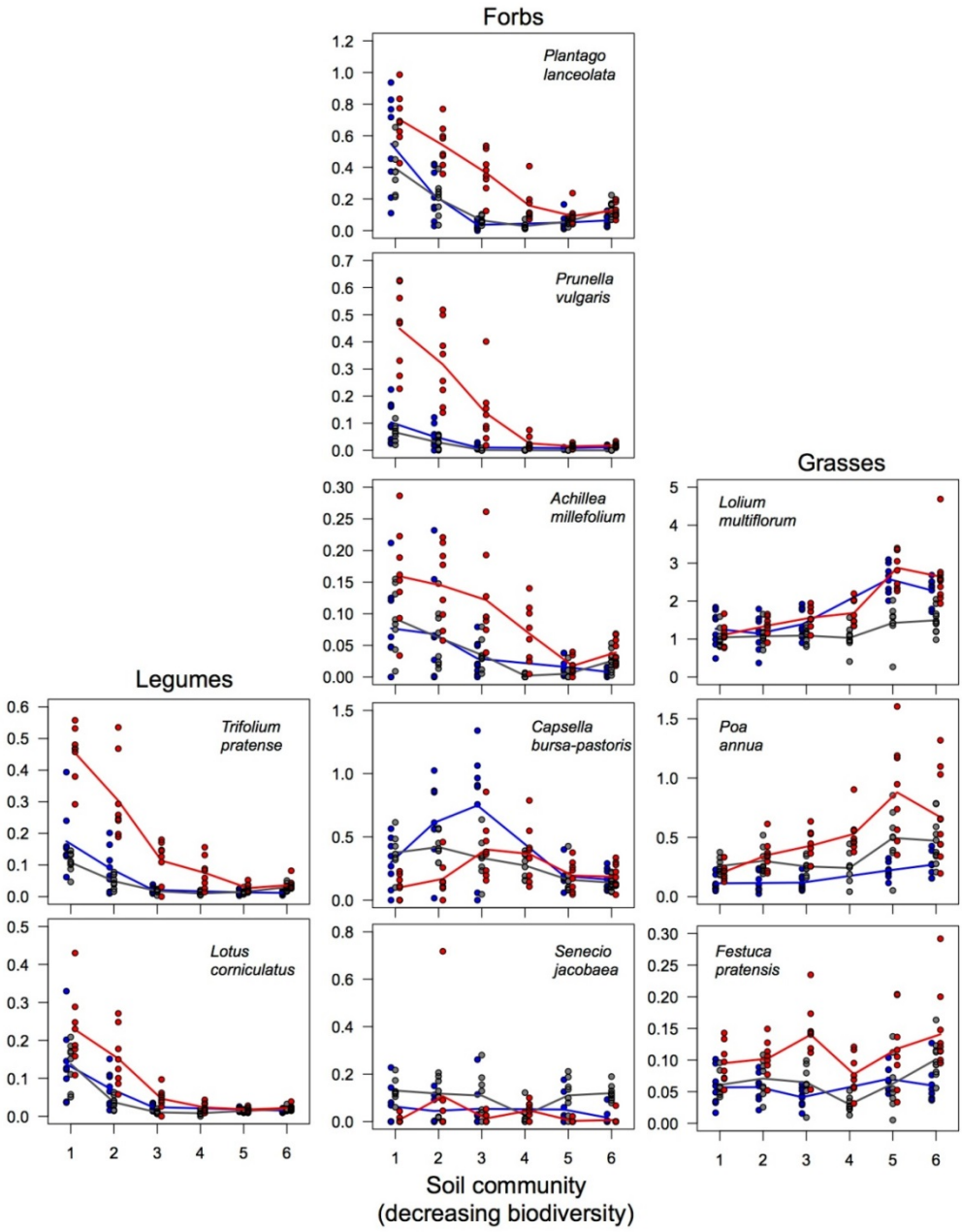


Fig S3. Data of plant species biomass (g) per individual in grassland microcosm with six different soil communities. The data for experiment 1 (blue lines and dots), experiment 2 after 12 weeks (grey lines and dots), and experiment 2 after 24 weeks (red lines and dots) are shown. Lines show the trend between consecutive means for each filter treatment. Points are staggered for clarity. Soil communities were established by filtering through different meshes: 1 ≤ 5000 μm, 2 ≤ 250 μm, 3 ≤ 50 μm, 4 ≤ 25 μm, 5 ≤ 10 μm, and 6 ≤ sterilized soil.

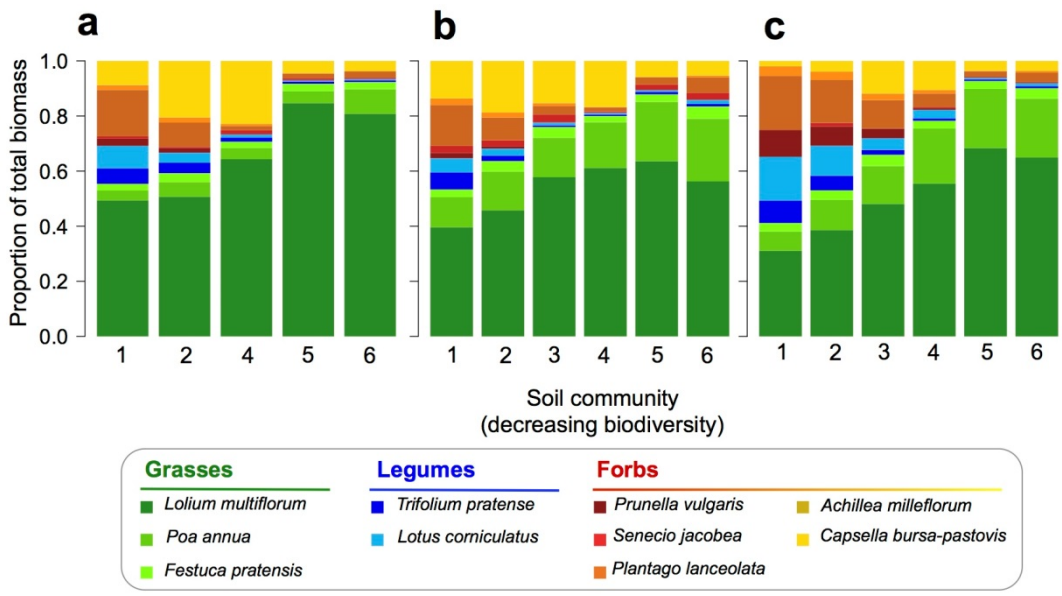


Fig S4. The proportion of the total biomass of each plant species in grassland microcosms with six different soil communities. The plant community composition is shown for (a) experiment 1, (b) experiment 2 after 12 weeks, and (c) experiment 2 at 24 weeks. Soil communities were established by filtering through different meshes: 1 ≤ 5000 μm, 2 ≤ 250 μm, 3 ≤ 50 μm, 4 ≤ 25 μm, 5 ≤ 10 μm, and 6 ≤ sterilized soil.

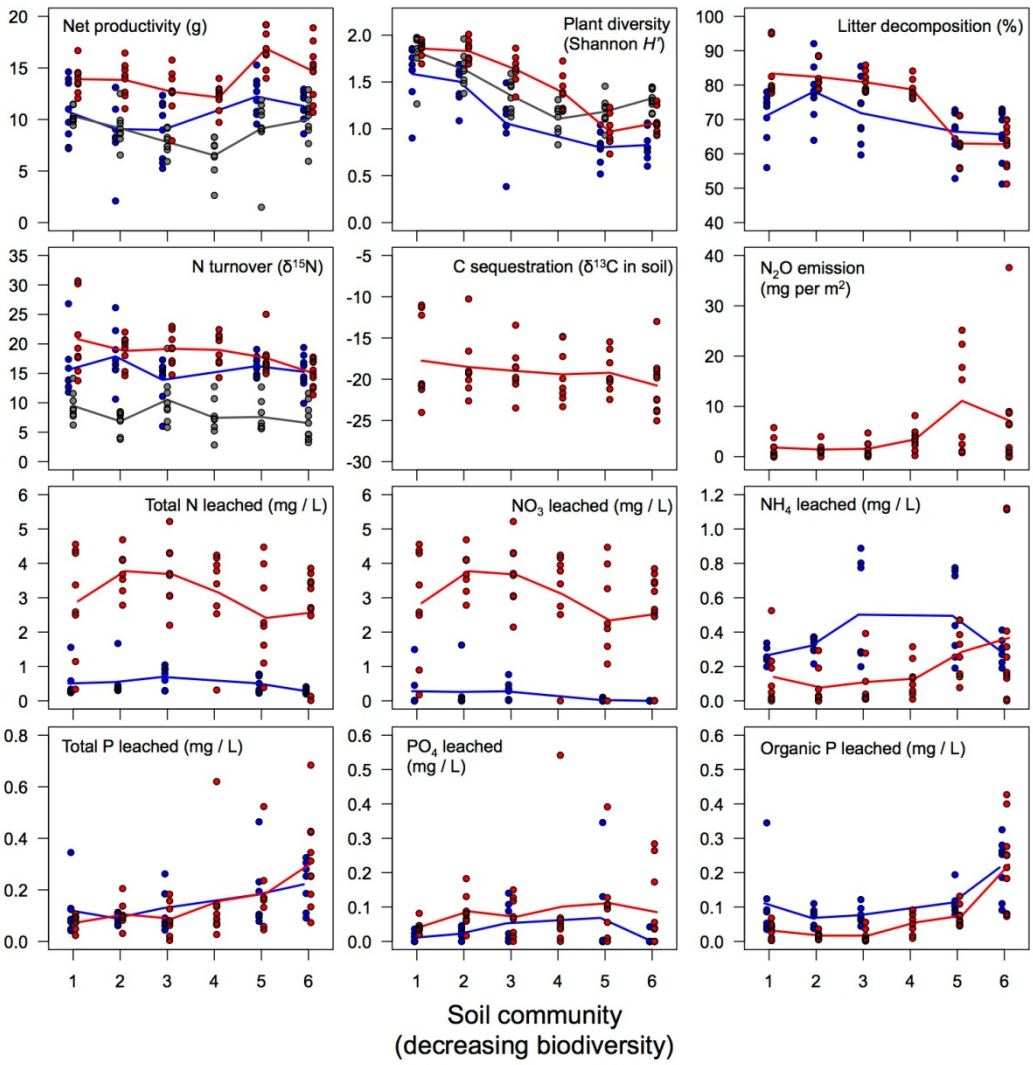


Fig S5. Data of the various ecosystem functions in grassland microcosms with six different soil communities. The data for experiment 1 (blue lines and dots), experiment 2 after 12 weeks (grey lines and dots), and experiment 2 after 24 weeks (red lines and dots) are shown. The leached PO_4 and organic P comprise the total P leached and the NO_3 and NH_4 leached comprise the total N leached. All N leaching data are expressed as log+1 transformed to improve clarity. N turnover ($\delta^{15}N$) values from experiment 1 (blue) are scaled by 10^1 for clarity. Lines show the trend between consecutive means for each filter treatment. Points are staggered for clarity. Soil communities were established by filtering through different meshes: 1 ≤ 5000 μm, 2 ≤ 250 μm, 3 ≤ 50 μm, 4 ≤ 25 μm, 5 ≤ 10 μm, and 6 ≤ sterilized soil. The average response of both experiments is shown in Fig. 2 in the main text.

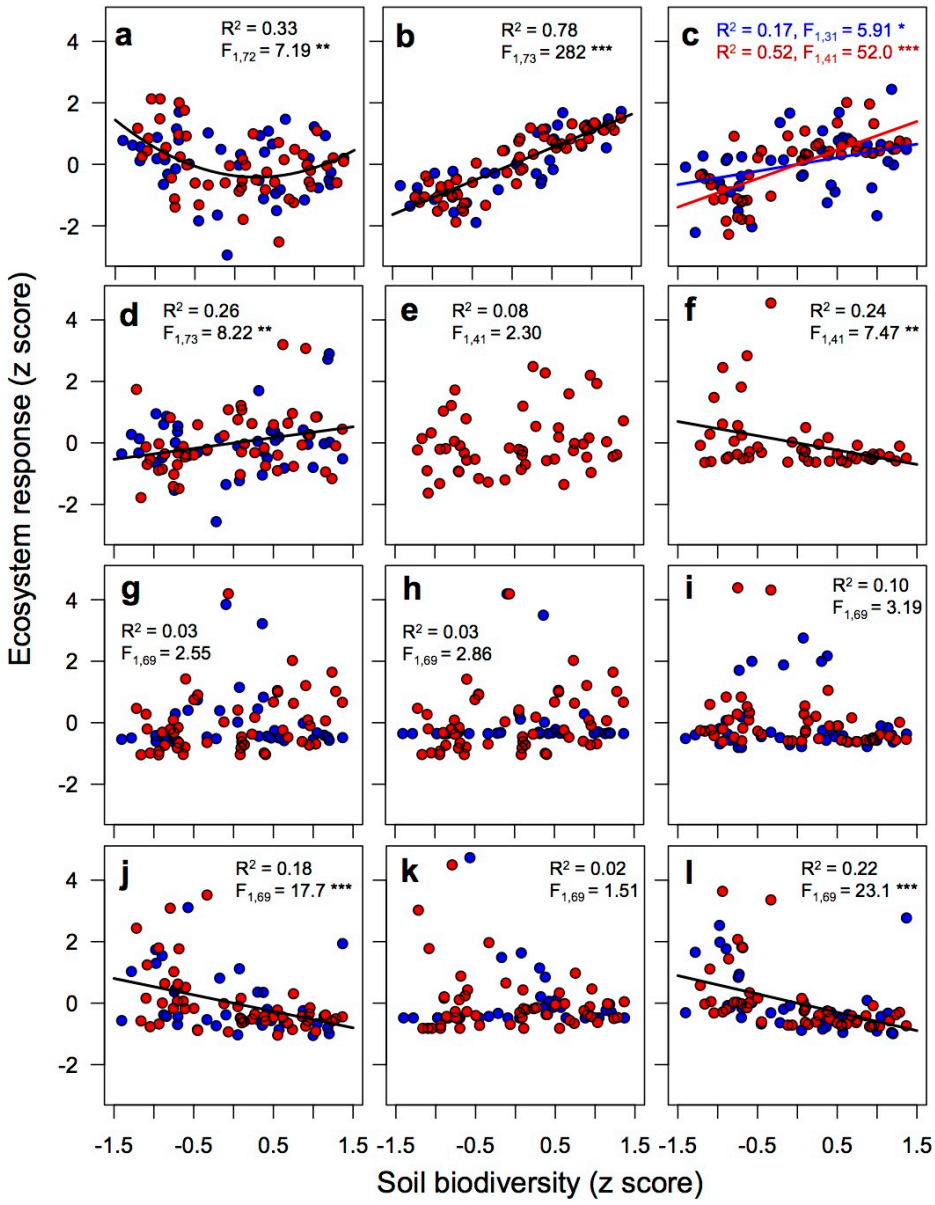


Fig S6. Standardized response of ecosystem functions to standardized average of soil biodiversity indicators. Data are from the final harvests of experiment 1 (blue) and experiment 2 (red). The panels are (a) plant productivity, (b) plant diversity, (c) litter decomposition, (d) N turnover, (e) carbon sequestration, (f) N_2O emission, (g) total N leached, (h) NO_3 leached, (i) NH_4 leached, (j) total P leached, (k) PO_4 leached, and (l) organic P leached. Regression lines are shown where relationships were found to be significant. Different regression lines for each trial are shown for litter decomposition (c) since the relationship to soil biodiversity was dependent on the trial (trial x soil biodiversity interaction: $F_{1,72} = 5.02$, $P = 0.03$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

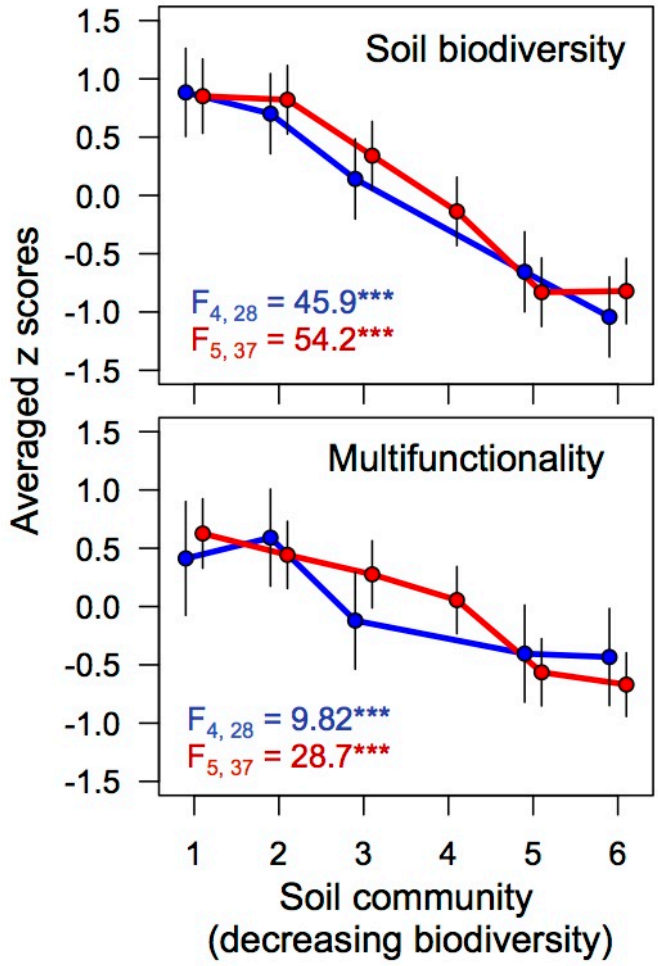
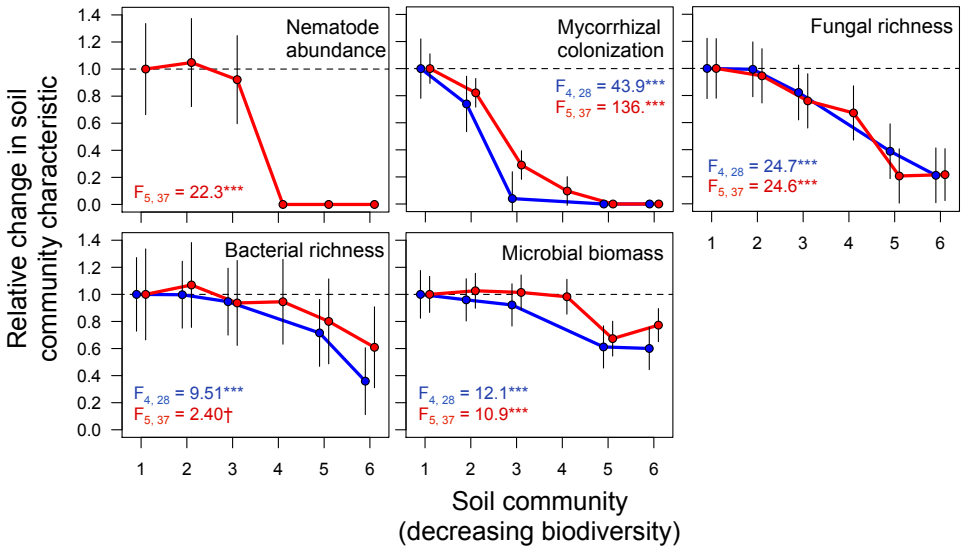


Fig S7. Average soil biodiversity and ecosystem multifunctionality in grassland microcosms for the six different soil community treatments. Blue indicates results from experiment 1 and red results from experiment 2. Means and 95 % confidence intervals are estimated from the ANOVA model. Soil communities were established by filtering through different meshes: 1 ≤ 5000 μm, 2 ≤ 250 μm, 3 ≤ 50 μm, 4 ≤ 25 μm, 5 ≤ 10 μm, and 6 ≤ sterilized soil. *** P < 0.001


Relative change in soil community characteristic
Nematode abundance
$F_{5, 37}$ = 22.3***
Mycorrhizal colonization
$F_{4, 28}$ = 43.9***
$F_{5, 37}$ = 136.***
Fungal richness
$F_{4, 28}$ = 24.7***
$F_{5, 37}$ = 24.6***
Bacterial richness
$F_{4, 28}$ = 9.51***
$F_{5, 37}$ = 2.40†
Microbial biomass
$F_{4, 28}$ = 12.1***
$F_{5, 37}$ = 10.9***
Soil community
(decreasing biodiversity)

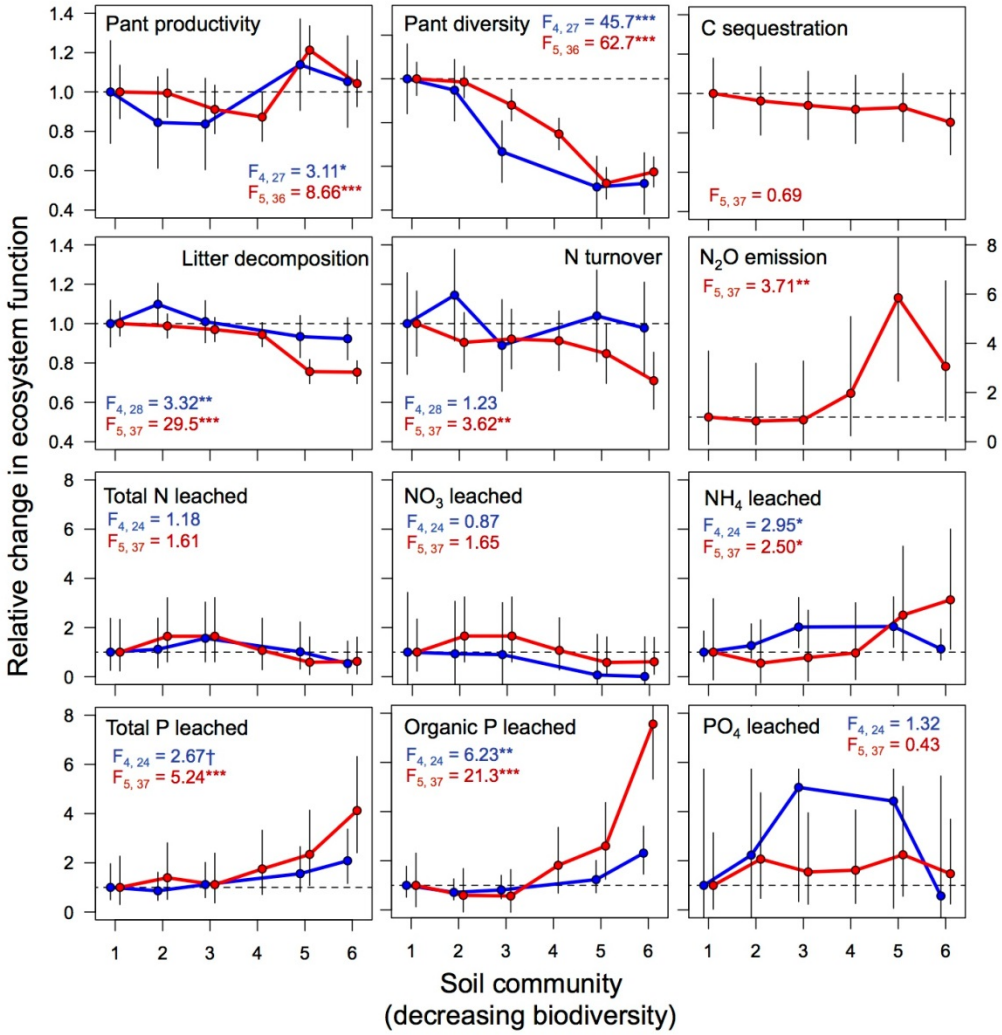

Relative change in ecosystem function
Pant productivity
$F_{4, 27}$ = 3.11*
$F_{5, 36}$ = 8.66***
Pant diversity
$F_{4, 27}$ = 45.7***
$F_{5, 36}$ = 62.7***
C sequestration
$F_{5, 37}$ = 0.69
Litter decomposition
$F_{4, 28}$ = 3.32**
$F_{5, 37}$ = 29.5***
N turnover
$F_{4, 28}$ = 1.23
$F_{5, 37}$ = 3.62**
N_2O emission
$F_{5, 37}$ = 3.71**
Total N leached
$F_{4, 24}$ = 1.18
$F_{5, 37}$ = 1.61
NO_3 leached
$F_{4, 24}$ = 0.87
$F_{5, 37}$ = 1.65
NH_4 leached
$F_{4, 24}$ = 2.95*
$F_{5, 37}$ = 2.50*
Total P leached
$F_{4, 24}$ = 2.67†
$F_{5, 37}$ = 5.24***
Organic P leached
$F_{4, 24}$ = 6.23**
$F_{5, 37}$ = 21.3***
PO_4 leached
$F_{4, 24}$ = 1.32
$F_{5, 37}$ = 0.43
Soil community
(decreasing biodiversity)

Fig S8. Relative changes in soil community characteristics and ecosystem functions by experimental trial in grassland microcosms inoculated with six different soil communities. Blue indicates results from experiment 1 and red results from experiment 2. Means and 95 % confidence intervals from the ANOVA model are expressed as a ratio of the 5000 μm soil community. Confidence intervals are for the comparison to 1 (no change in ecosystem functioning from the soil community 1, dashed line). Soil communities were established by filtering through different meshes: 1 ≤ 5000 μm, 2 ≤ 250 μm, 3 ≤ 50 μm, 4 ≤ 25 μm, 5 ≤ 10 μm, and 6 ≤ sterilized soil. Note the upper limits of the confidence intervals for PO_4 leaching data from experiment 1 (blue) are not shown for clarity. The high level of variation in PO_4 leaching data in experiment 1 results from the values frequently occurring below the detection limit (see Supplementary Figure 4). † P < 0.05, * P < 0.05, ** P < 0.01, *** P < 0.001.

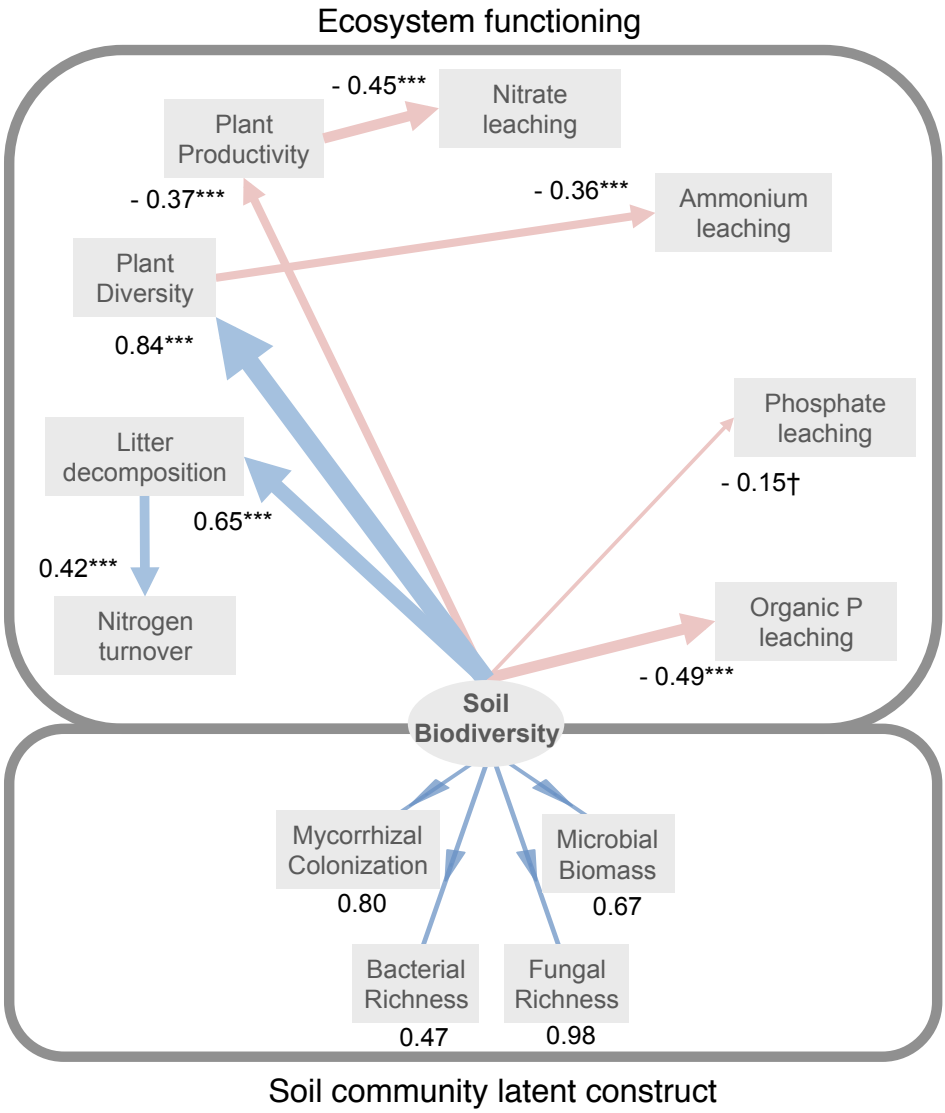


Fig S9. The path model illustrating the direct and indirect effects of soil biodiversity on various ecosystem functions are shown for the most parsimonious model (see Table S2, Goodness of Fit = 0.38). The model was constructed using the pooled data of variables common to both experiments. Larger path coefficients are reflected in the width of the arrow with blue indicating a positive effect and red a negative effect. The loadings for each of the soil community characteristics that create the soil biodiversity latent variable are shown the lower panel (the soil community latent construct). Within the ecosystem functioning compartment path coefficients that differ significantly from 0 are, are indicated by *P = < 0.05, **P = < 0.01, ***P = < 0.001, †P = 0.16; significance is based on 1000 resampled bootstraps.

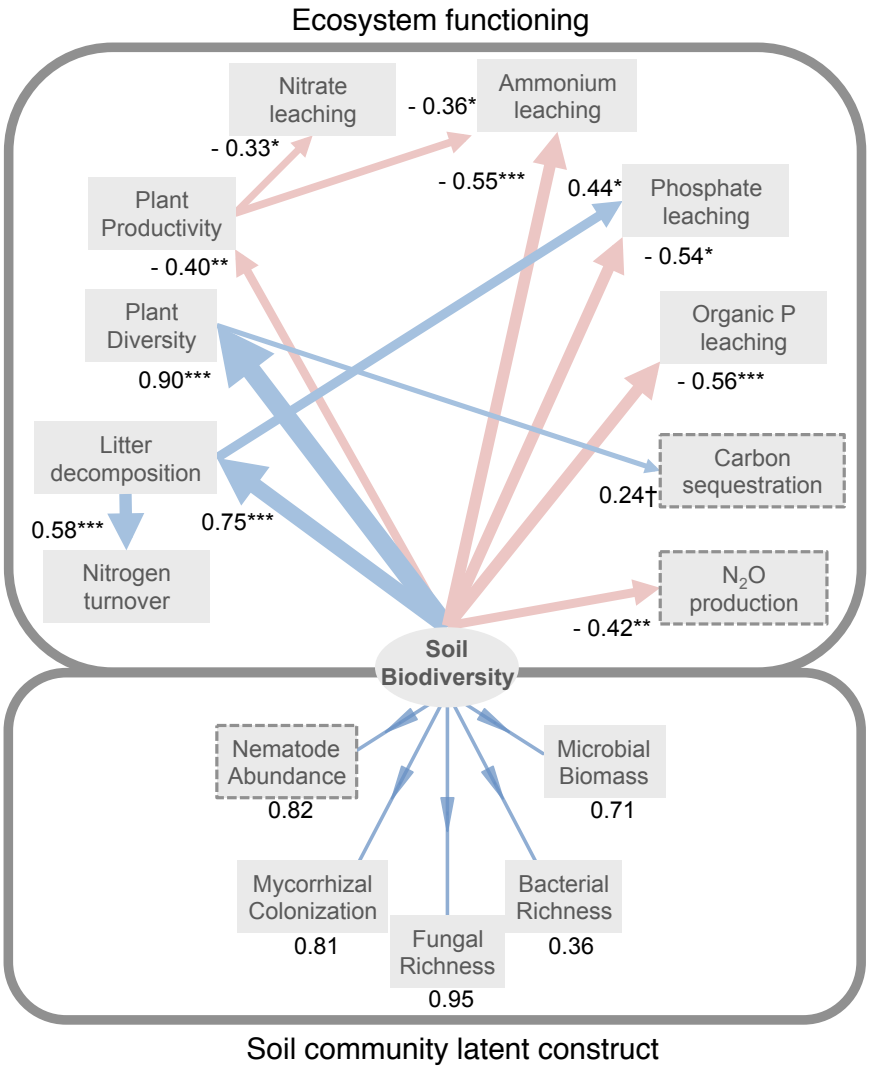


Fig S10. The path model illustrating the direct and indirect effects of soil biodiversity on various ecosystem functions are shown for the most parsimonious model (see Table S3, Goodness of Fit = 0.41). The model was constructed using only the data in experiment 2 to assess variables that were not measured in experiment 1. Larger path coefficients are reflected in the width of the arrow with blue indicating a positive effect and red a negative effect. The loadings for each of the soil community characteristics that create the soil biodiversity latent variable are shown the lower panel (the soil community latent construct). Within the ecosystem functioning compartment path coefficients that differ significantly from 0 are, are indicated by *P = < 0.05, **P = < 0.01, ***P = < 0.001, †P = 0.16; significance is based on 1000 resampled bootstraps.

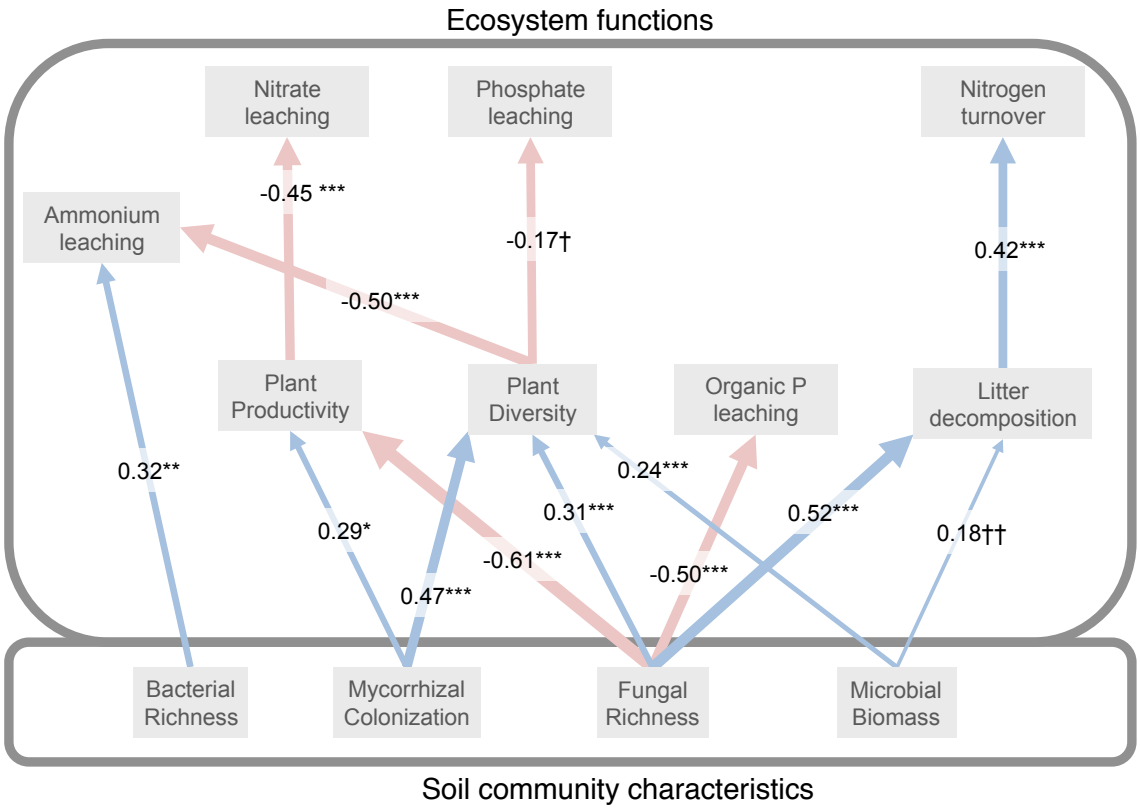


Fig S11. The most parsimonious path model illustrating the direct and indirect effects of the individual soil community characteristics on the various ecosystem functions is shown. The model was constructed using the pooled data of variables common to both experiments. Larger path coefficients are reflected in the width of the arrow with blue indicated a positive effect and red a negative effect. Significance differences in path coefficients from 0, based on 1000 resampled bootstrap t-test, are indicated by *P = < 0.05, **P = < 0.01, ***P = < 0.001, †P = 0.12, †† P = 0.09.

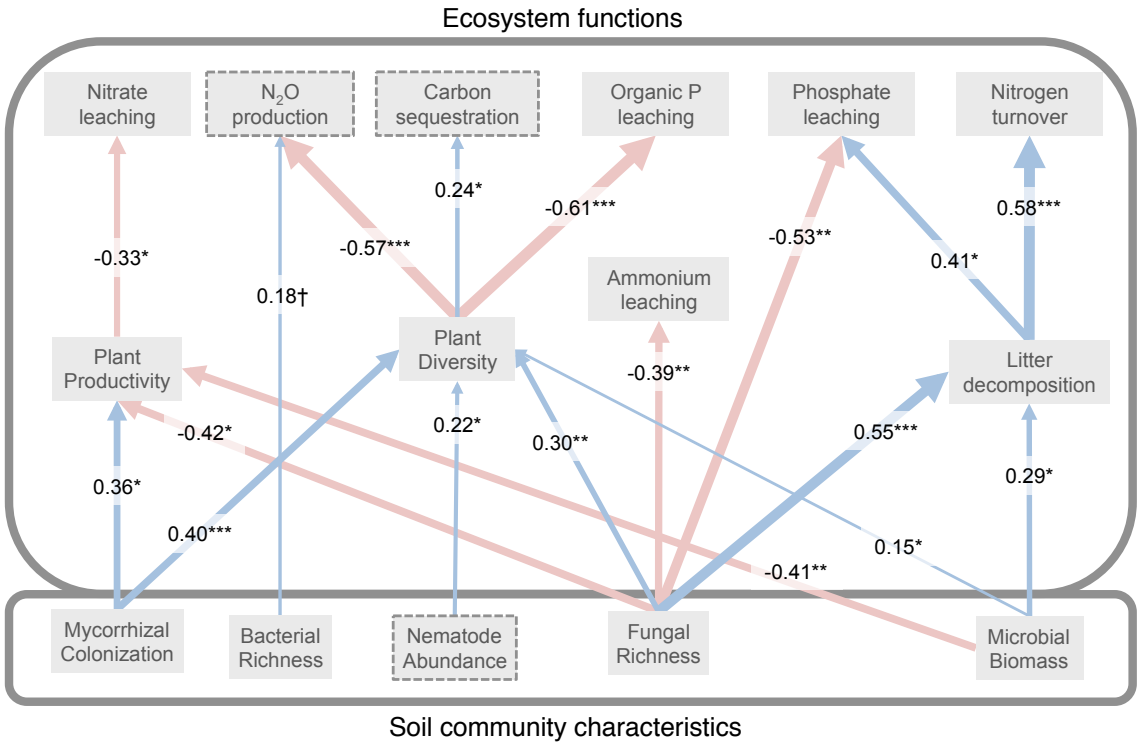


Fig S12. The most parsimonious path model illustrating the direct and indirect effects of the individual soil community characteristics on the various ecosystem functions is shown. The model was constructed using only the data in experiment 2 to assess variables that were not measured in experiment 1. Larger path coefficients are reflected in the width of the arrow with blue indicated a positive effect and red a negative effect. Significance differences in path coefficients from 0, based on 1000 resampled bootstrap t-test, are indicated by *P = < 0.05, **P = < 0.01, ***P = < 0.001, †P = 0.12, †† P = 0.09.

SI: Tables

Table S1. ANOVA results for the variation in ecosystem characteristics as explained by differences between experiments and soil community treatments. Data were assessed for whether the variation among soil community treatments depended on the harvest (soil community "By harvest" interaction term as shown in column two and three) and the overall effect of soil communities for all harvest and trial data "Pooled" as shown in column four and five. The assessment among harvests was done using data from the five soil community treatments common to both trials, while "Pooled" included all soil community treatments. See fig. S6 and S7 for ANOVA results for each trial individually. The results show that most ecosystem characteristics were strongly affected by differences in soil communities irrespective of harvest, while some ecosystem characteristics (plant diversity, decomposition, N turnover, NH_4 leached) additionally also differed between the harvests.

	By harvest		Pooled	
Ecosystem characteristic	**df**	**F**	**df**	**F**
Soil community characteristics				
Mycorrhiza	4, 57	2.49	5, 68	122 ***
Nematodes			5, 37	22.3 ***
Fungi	§8, 94	0.37	5, 111	74.5 ***
Bacteria	§8, 94	0.46	5, 111	18.3 ***
Microbial biomass	§8, 94	0.37	5, 111	16.6 ***
Soil biodiversity	§8, 94	1.01	5, 111	91.4 ***
Ecosystem functions				
Primary productivity	§8, 93	0.89	5, 110	13.6 ***
Plant diversity	§8, 93	3.89 ***	5, 110	55.7 ***
Decomposition	4, 58	3.05 *	5, 69	15.0 ***
N turnover	§8, 94	2.21 *	5, 111	2.58 *
C sequestration			5, 37	0.68
N_2O emissions			5, 37	3.71 **
Total N leached	4, 54	0.50	5, 65	2.30
Nitrate leached	4, 54	0.43	5, 65	2.25
Ammonium leached	4, 54	2.66 *	5, 65	2.27
Total P leached	4, 54	0.40	5, 65	7.00 ***
Organic P leached	4, 54	0.30	5, 65	22.1 ***
Phosphate leached	4, 54	0.50	5, 65	1.03
Multifunctionality index	4, 58	2.23	5, 69	69.1 ***

* P < 0.05, ** P < 0.01, *** P < 0.001, df = degrees of freedom, §Note: the 8 numerator df of some ecosystem characteristics indicate they were also measured at the 12 week harvest point in experiment 2, and thus are assessed across all three harvest points. The 4 numerator df of some ecosystem characteristics indicate they were only measured at the final harvest.

Table S2. Coefficients for direct, indirect, and the bootstrap estimated total effects for the effect of soil biodiversity on each ecosystem function using pooled data for all common variables. Coefficients for paths among ecosystem functions that were included in the final model are also listed (Goodness of Fit = 0.38). Path origins are in italics followed by the response variables listed beneath. Lower and upper 95 % bootstraped confidence intervals are shown in parenthesizes for the total effect (combined direct and indirect effects).

Pooled	**Direct**	**Indirect**	**Total**
Soil biodiversity			
Net productivity	-0.37	0.00	-0.37 (-0.54, -0.18)
Plant diversity	0.84	0.00	0.83 (0.76, 0.89)
Ammonium leach.	0.00	-0.30	-0.30 (-0.43, -0.17)
Nitrate leach.	0.00	0.17	0.17 (0.06, 0.29)
Phosphate leach.	-0.15	0.00	-0.16 (-0.31, 0.08)
Organic P leach.	-0.49	0.00	-0.51 (-0.67, -0.3)
Decomposition	0.65	0.00	0.65 (0.52, 0.75)
N turnover	0.00	0.27	0.27 (0.10, 0.42)
Decomposition			
N turnover	0.42	0.00	0.41 (0.17, 0.62)
Net productivity			
Nitrate leach.	-0.45	0.00	-0.44 (-0.64, -0.19)
Plant diversity			
Ammonium leach.	-0.36	0.00	0.36 (-0.50, -0.20)

Table S3. Coefficients for direct, indirect, and the bootstrap estimated total effects for the effect of soil biodiversity on each ecosystem function. Coefficients for paths among ecosystem functions are were included in the final model are also listed (Goodness of Fit = 0.41). The model was constructed using all data collected during the second experiment; which includes nematode abundance, C sequestration, and N_2O emission that were not included in the model using pooled data from both experiments. Path origins are in italics followed by the response variables listed beneath. Lower and upper 95 % bootstraped confidence intervals are shown in parenthesizes for the total effect (combined direct and indirect effects).

Experiment 2 only	**Direct**	**Indirect**	**Total**
Soil biodiversity			
Net productivity	-0.40	0.00	-0.40 (-0.60, -0.16)
Plant diversity	0.90	0.00	0.89 (0.84, 0.93)
Ammonium leach	-0.55	0.14	-0.43 (-0.61, -0.24)
Nitrate leach.	0.00	0.13	0.14 (0.01, 0.29)
Phosphate leach.	-0.54	0.33	-0.21 (-0.43, 0.11)
Organic P leach.	-0.56	0.00	-0.59 (-0.70, -0.48)
Decomposition	0.76	0.00	0.76 (0.67, 0.84)
N turnover	0.00	0.44	0.44 (0.27, 0.58)
C sequestration	0.00	0.21	0.21 (-0.04, 0.42)
N_2O emission	-0.42	0.00	-0.44 (-0.64, -0.20)
Decomposition			
N turnover	0.58	0.00	0.57 (0.36, 0.73)
Phosphate leach.	0.44	0.00	0.42 (-0.15, 0.80)
Net productivity			
Ammonium leach	-0.36	0.00	-0.36 (-0.58, -0.1)
Nitrate leach.	-0.33	0.00	-0.33 (-0.57, -0.06)
Plant diversity			
C sequestration	0.24	0.00	0.24 (-0.04, 0.47)

Table S4. Coefficients for direct, indirect, and the bootstrap estimated total effects for the effect of individual soil community characteristics on each ecosystem function using pooled data for all common variables between the two experiments. Coefficients for paths among ecosystem functions that were included in the final model are also listed. Path origins are in italics followed by the response variables listed beneath. Lower and upper 95 % bootstraped confidence intervals are shown in parenthesizes for the total effect (combined direct and indirect effects).

Pooled	**Direct**	**Indirect**	**Total**
Mycorrhiza			
Net productivity	0.29	0.00	0.28 (0.00, 0.54)
Plant diversity	0.47	0.00	0.47 (0.30, 0.62)
Ammonium leach	0.00	-0.23	-0.24 (-0.36, -0.12)
Nitrate leach.	0.00	-0.13	-0.12 (-0.27, 0.00)
Phosphate leach.	0.00	-0.08	-0.08 (-0.16, 0.01)
Microbial abundance			
Decomposition	0.18	0.00	0.18 (-0.05, 0.39)
N turnover	0.00	0.07	0.08 (-0.02, 0.20)
Plant diversity	0.24	0.00	0.24 (0.11, 0.36)
Ammonium leach.	0.00	-0.12	-0.12 (-0.20, -0.05)
Phosphate leach.	0.00	-0.04	-0.04 (-0.08, 0.00)
Fungal richness			
Decomposition	0.52	0.00	0.51 (0.34, 0.67)
N turnover	0.00	0.22	0.21 (0.08, 0.35)
Net productivity	-0.61	0.00	-0.60 (-0.82, -0.32)
Plant diversity	0.31	0.00	0.31 (0.16, 0.46)
Ammonium leach	0.00	-0.15	-0.16 (-0.27, -0.06)
Nitrate leach.	0.00	0.27	0.26 (0.10, 0.46)
Phosphate leach.	0.00	-0.05	-0.05 (-0.11, 0.01)
Organic P leach.	-0.50	0.00	-0.50 (-0.65, -0.30)
Bacterial richness			
Ammonium leach.	0.32	0.00	0.33 (0.10, 0.52)
Decomposition			
N turnover	0.42	0.00	0.41 (0.17, 0.61)
Net productivity			
Nitrate leach.	-0.45	0.00	-0.43 (-0.62, -0.20)

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Plant diversity			
Ammonium leach.	-0.5	0.00	-0.51 (-0.68, -0.30)
Phosphate leach.	-0.17	0.00	-0.16 (-0.32, 0.02)

Table S5. Coefficients for direct, indirect, and the bootstrap estimated total effects for the effect of the individual soil community characteristics on each ecosystem function. Coefficients for paths among ecosystem functions are were included in the final model are also listed. The model was constructed using all data collected during the second experiment; which includes nematode abundance, C sequestration, and N_2O emission that were not included in the model using pooled data from both experiments. Path origins are in italics followed by the response variables listed beneath. Lower and upper 95 % bootstraped confidence intervals are shown in parenthesizes for the total effect (combined direct and indirect effects).

Experiment 2 only	**Direct**	**Indirect**	**Total**
Nematode abundance			
Plant diversity	0.22	0.00	0.21 (0.01, 0.36)
Organic P leach.	0.00	-0.13	-0.13 (-0.23, -0.01)
C sequestration	0.00	0.05	0.05 (-0.01, 0.12)
N_2O emission	0.00	-0.13	-0.12 (-0.23, -0.01)
Mycorrhiza			
Net productivity	0.36	0.0	0.36 (0.01, 0.68)
Plant diversity	0.40	0.00	0.41 (0.21, 0.62)
Nitrate leach.	0.00	-0.12	-0.11 (-0.26, 0.00)
Organic P leach.	0.00	-0.24	-0.25 (-0.38, -0.13)
C sequestration	0.00	0.10	0.10 (-0.01, 0.24)
N_2O emission	0.00	-0.23	-0.23 (-0.41, -0.09)
Microbial abundance			
Decomposition	0.29	0.00	0.29 (0.05, 0.51)
N turnover	0.00	0.17	0.17 (0.03, 0.30)
Net productivity	-0.41	0.00	-0.42 (-0.66, -0.15)
Plant diversity	0.15	0.00	0.15 (0.00, 0.30)
Nitrate leach.	0.00	0.14	0.14 (0.01, 0.32)
Phosphate leach.	0.00	0.12	0.11 (-0.01, 0.27)
Organic P leach.	0.00	-0.09	-0.09 (-0.19, 0.00)
C sequestration	0.00	0.04	0.03 (-0.01, 0.09)
N_2O emission	0.00	-0.09	-0.08 (-0.18, 0.00)

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Fungal richness			
Decomposition	0.55	0.00	0.55 (0.34, 0.77)
N turnover	0.00	0.32	0.31 (0.16, 0.49)
Net productivity	-0.42	0.00	-0.41 (-0.75, 0.01)
Plant diversity	0.30	0.00	0.30 (0.16, 0.45)
Ammonium leach.	-0.39	0.00	-0.40 (-0.55, -0.20)
Nitrate leach.	0.00	0.14	0.13 (-0.01, 0.29)
Phosphate leach.	-0.53	0.23	-0.30 (-0.51, -0.03)
Organic P leach.	0.00	-0.18	-0.19 (-0.28, -0.10)
C sequestration	0.00	0.07	0.07 (-0.01, 0.15)
N_2O emission	0.00	-0.17	-0.17 (-0.29, -0.07)
Bacterial richness			
N_2O emission	0.18	0.00	0.18 (-0.10, 0.45)
Decomposition			
N turnover	0.58	0.00	0.57 (0.36, 0.73)
Phosphate leach.	0.41	0.00	0.38 (-0.02, 0.75)
Net productivity			
Nitrate leach.	-0.33	0.00	-0.33 (-0.57, -0.05)
Plant diversity			
Organic P leach.	-0.61	0.00	-0.61 (-0.73, -0.51)
C sequestration	0.24	0.00	0.24 (-0.05, 0.46)
N_2O emission	-0.57	0.00	-0.57 (-0.78, -0.31)

Summary

Agricultural practices as applied in many parts of the world are based on high external fertilizer inputs. This assures high crop yields and provides the nutritional basis for huge parts of the global population but also leads to strong impacts on the environment. Moreover, it is expected that fertilizer resources will become more limited in the future. Only about half of the nutrients applied are taken up by crop plants, while a big portion remains in soil and is prone to be lost from the plant soil system. Nutrient losses of N and P through leaching from agricultural fields are considered a main cause for water eutrophication. N can be transformed into gases like N_2O, a strong greenhouse gas also involved in the destruction of the stratospheric ozone layer. Furthermore, increased amounts of nutrients circulating through the environmental system can decrease biodiversity and harm the integrity of natural ecosystems adapted to low nutrient concentrations. There is, hence, a strong need to reduce nutrient losses from and to enhance the nutrient use efficiency of agricultural systems to be able to spare limiting resources, to reduce negative environmental impacts and to assure sufficient yields to be able to meet the demands of a growing global human population.

Soil biota conduct most nutrient transformation processes in the soil and determine e.g. the rates of nutrient mineralization from organic matter and the chemical forms and, hence, plant availability of important plant nutrients. However, little is known about their potential to enhance efficient nutrient cycling in agricultural systems including improved plant nutrition and reduced nutrient losses to the environment.

Arbuscular mycorrhizal fungi (AMF) are a globally dispersed group of soil microorganisms forming symbiotic relationships with the majority of land plants and are known to improve plant nutrition with important nutrients. There are also indications that these fungi can reduce nutrient leaching losses from soil. These positive effects of AMF appear, however, to be context dependent and their potential to enhance the nutrient use efficiency in agricultural cropping systems is not well known.

Furthermore it remains unknown, whether these fungi affect gaseous emissions of N, e.g. though the microbial process of denitrification.

This thesis addresses the question whether the plant symbiotic AMF have the potential to enhance sustainable nutrient cycling in soil resulting in improved plant performance and in reduced nutrient losses from the plant-soil system and alleviating agricultural cropping systems from the strong dependency on excessive inputs of external fertilizers.

In Chapter 1, using data from 2 independent but complementary experiments, I showed that AMF have the potential to reduce emissions of the greenhouse gas N_2O, representing a potential loss of nutrients as well as a substance contributing to environmental hazards like global warming and the destruction of the stratospheric ozone layer. In Chapter 2, the influence of AMF on overall N and P cycling including leaching losses and losses as N_2O in experimental grasslands of different environmental conditions was assessed. I found that the effects of AMF on nutrient leaching and plant nutrition differed with soil conditions. The influence of AMF on plant P nutrition and the reduction of P leaching seemed to be more consistent than effects on plant N nutrition and N leaching. Again, I found reduced fluxes of N_2O in presence of AMF. Chapter 3 specifically addressed N cycling in dependence of AMF. The results reveal a great potential of AMF to improve plant N nutrition, reduce N leaching losses and N_2O emissions. Moreover, the data suggest that AMF might enhance denitrification efficiency resulting in lower N_2O and higher N_2 emissions. These results could have important implications for the management of global N cycling. In chapter 4, the effects of AMF and other soil organisms on nutrient cycling were tested in an agriculturally more relevant setting in a crop rotation in an outdoor lysimeter experiment. Over a period of nearly two years, data on nutrient leaching and plant growth, nutrition and agricultural yields was collected. I found that soil biota including AMF strongly reduced N leaching losses and improved crop growth and nutrition. P leaching was higher with soil biota but was low compared to the strong increases in plant P contents.

Overall, the results obtained during four years of research reveal a great potential of AMF and other soil biota to enhance the nutrient use efficiency and, hence the sustainability of cropping systems and provide a mechanistic basis that helps to explain observations made in field based research.

The implementation of agricultural management practices that promote AMF and other soil organisms could be an important step towards a sustainable agriculture that is able to meet the demands of the present without compromising the ability of meeting the demands of future generations.

Zusammenfassung

Landwirtschaftliche Praktiken wie sie heute in grossen Teilen der Welt angewandt werden, basieren auf hohen Nährstoffeinträgen durch Düngemittel. Dies gewährleistet hohe Erträge, welche die Ernährungsgrundlage für grosse Teile der Weltbevölkerung bilden, aber bringt auch grosse Umweltprobleme mit sich. Ausserdem wird erwartet, dass die Verfügbarkeit von Düngemittel-ressourcen in Zukunft eingeschränkt sein könnte.

Ungefähr die Hälfte der applizierten Düngemittel wird von den Ackerkulturen aufgenommen, während ein grosser Teil im Boden verbleibt und potentiell der Gefahr unterliegt aus dem Boden-Pflanze-System verloren zu gehen. Auswaschungsverluste von N und P aus Ackerflächen werden als Hauptursache für Gewässereutrophierung angesehen. N kann in gasförmige Verbindungen wie N2O umgewandelt werden, ein starkes Treibhausgas, dass auch an der Zerstörung der Ozonschicht in der Stratosphäre beteiligt ist. Ausserdem können erhöhte Mengen an Nährstoffen die sich durch das Umweltsystem bewegen die Biodiversität verringern und die Funktionen natürlicher Ökosysteme, die an niedrige Nährstoffkonzentrationen angepasst sind beeinträchtigen. Es ist daher enorm wichtig, die Nährstoffeffizienz von landwirtschaftlichen Systemen zu erhöhen und Nährstoffverluste zu reduzieren um Ressourcen zu schonen, negative Umweltauswirkungen zu verringern und ausreichende Erträge zu erreichen um die Bedürfnisse einer wachsenden Weltbeförderung zu befriedigen.

Bodenlebewesen führen einen Grossteil der Nährstoffumwandlungen im Boden durch und bestimmen z.B. die Raten der Nährstoffmineralisation aus organischer Substanz und die chemische Form und somit auch die Pflanzenverfügbarkeit wichtiger Pflanzennährstoffe. Ihr Potential effiziente Nährstoffkreisläufe in landwirtschaftlichen Systemen zu fördern und somit die Pflanzenernährung zu erhöhen und Nährstoffverluste zu verringern ist jedoch noch nicht hinreichend bekannt.

Arbuskuläre Mykorrhizapilze (AMP) sind eine weltweit verbreitete Gruppe von Bodenmikroorganismen, die symbiotische Beziehungen mit der Mehrheit aller Landpflanzen

eingehen und die für Ihre positive Wirkung auf die Pflanzenernährung mit wichtigen Nährstoffen bekannt sind. Es gibt ausserdem Hinweise darauf, dass diese Pilze Nährstoffverluste durch Auswaschung aus dem Boden verringern können. Diese positiven Eigenschaften von AMP scheinen jedoch Kontextanhängig zu sein und ihr Potential die Nährstoffeffizienz in Ackerbausystemen zu erhöhen ist nicht gut untersucht. Ausserdem ist bislang unbekannt, wie diese Pilze gasförmige Verluste von N, z.B. durch den mikrobiellen Prozess der Denitrifikation, beeinflussen.

Diese Dissertation bearbeitet die Frage, ob die Pflanzensymbiotischen AMP das Potential besitzen nachhaltige Nährstoffkreisläufe im Boden mit dem Ergebnis zu erhöhen, dass die Pflanzenernährung verbessert wird, während Nährstoffverluste aus dem Boden verringert werden, um so die Abhängigkeit landwirtschaftlicher Systeme von externen Nährstoffeinträgen zu reduzieren.

In Kapitel 1 benutze ich Daten aus 2 unabhängigen, aber komplementären Experimenten, um zu zeigen, dass AMP das Potential besitzen Emissionen des starken Treibhausgases N2O zu reduzieren, welche sowohl einen potentiellen Nährstoffverlust darstellen, als auch zu Umweltproblemen wie globaler Erwärmung und der Zerstörung der Ozonschicht in der Stratosphäre beitragen.

In Kapitel 2 untersuche ich den Gesamteinfluss von AMP auf die Kreisläufe von N und P, inklusive Auswaschungsverlusten und N2O Emissionen in experimentellen Grasländern mit unterschiedlichen Umweltbedingungen. Die Effekte von AMP auf die Nährstoffauswaschung und Pflanzenernährung unterschieden sich unter verschieden Bodenbedingungen. Der AMP Einfluss auf die Pflanzenversorgung mit P und die Verringerung der P Auswaschung scheinen konsistenter zu sein als die Effekte auf die Pflanzenversorgung mit N und Auswaschungsverluste von N. N2O Flüsse waren in Anwesenheit von AMP reduziert.

Kapitel 3 beschäftigt sich speziell mit dem Einfluss von AMP auf den N Zyklus. Die Ergebnisse zeigen ein grosses Potential von AMP die Pflanzenernährung mit N zu verbessern, sowie N Auswaschungsverluste und N2O Emissionen zu reduzieren. Ausserdem deuten die Daten an, dass AMP die Effizienz der Denitrifikation erhöhen könnten, was zu niedrigen N2O aber erhöhten N2

Emissionen führen würde. Diese Ergebnisse könnten wichtige Konsequenzen für das Management globaler N Kreisläufe haben.

Kapitel 4 untersucht die Effekte von AMP und anderen Bodenorganismen auf Nährstoffkreisläufe in einem landwirtschaftlichen Zusammenhang in einer Fruchtfolge in einem Freiland-Lysimeter Experiment. Über einen Zeitraum von fast 2 Jahren wurden Daten über Nährstoffauswaschung, Pflanzenwachstum und –ernährung und landwirtschaftliche Erträge gesammelt. Bodenorgansimen inklusive AMP reduzierten die N Auswaschung enorm und erhöhten das Wachstum und Ernährung der Kulturen. Die P Auswaschung war in Anwesenheit von Bodenorganismen höher, war jedoch im Vergleich zu dem starken Anstieg der Pflanzen P Gehalte durch Bodenorganismen gering.

Die Ergebnisse, die in diesen 4 Forschungsjahren generiert wurden zeigen insgesamt ein grosses Potential von AMP und anderen Bodenorganismen die Nährstoffeffizienz und folglich die Nachhaltigkeit von Ackerbausystemen zu erhöhen. Sie bilden ausserdem eine mechanistische Grundlage, die behilflich ist, Beobachtungen aus Feldversuchen zu erklären.

Die Anwendung landwirtschaftlicher Praktiken, die AMP und andere Bodenlebewesen fördern, könnte ein wichtiger Schritt hin zu einer nachhaltigen Landwirtschaft sein, die fähig ist die gegenwärtigen Bedürfnisse zu stillen ohne die Befriedigung der Bedürfnisse zukünftiger Generationen zu gefährden.

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